CANADIAN THESES ON MICROFICHE

I.S.B.N.

THESES CANADIENNES SUR MICROFICHE

National Library of Canada
Collections Development Branch

Canadian Theses on
Microfiche Service

Ottawa, Canada
K1A 0N4

Bibliothèque nationale du Canada
Direction du développement des collections

Service des thèses canadiennes
sur microfiche

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de mauvaise qualité.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

NL-339 (r. 82/08)

Canada

0-315-08896-6

National Library of Canada
Canadian Theses Division
Ottawa, Canada
K1A 0N4

Bibliothèque nationale du Canada
Division des thèses canadiennes

6

56813

PERMISSION TO MICROFILM — AUTORISATION DE MICROFILMER

• Please print or type — Écrire en lettres moulées ou dactylographier

Full Name of Author — Nom complet de l'auteur

BARRY IAN CAPLAN

Date of Birth — Date de naissance	Country of Birth — Lieu de naissance
JULY 3, 1955	CANADA

Permanent Address — Résidence fixe

14 MARLOW PLACE
BRAMALEA, ONTARIO
L6S-2S3

Title of Thesis — Titre de la thèse

THE BIOCHEMICAL CHARACTERIZATION OF INTERLEUKIN 2

University — Université

UNIVERSITY OF ALBERTA

Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

DOCTOR OF PHILOSOPHY

Year this degree conferred — Année d'obtention de ce grade	Name of Supervisor — Nom du directeur de thèse
SPRING 1982	DR. V. Paetkau

Permission is hereby granted to the NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

L'autorisation est, par la présente, accordée à la BIBLIOTHÈQUE NATIONALE DU CANADA de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans l'autorisation écrite de l'auteur.

Date	Signature
December 30, 1981	Barry Caplan

NL-91 (4/77)

THE UNIVERSITY OF ALBERTA

THE BIOCHEMICAL CHARACTERIZATION OF INTERLEUKIN 2

by

© BARRY CAPLAN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

IN

BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

SPRING 1982

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR BARRY CAPLAN

TITLE OF THESIS THE BIOCHEMICAL CHARACTERIZATION OF INTERLEUKIN 2

DEGREE FOR WHICH THESIS WAS PRESENTED DOCTOR OF PHILOSOPHY

YEAR THIS DEGREE GRANTED SPRING 1982

Permission is hereby granted to THE UNIVERSITY OF ALBERTA LIBRARY to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

(SIGNED) Barry Caplan

PERMANENT ADDRESS:

14 Marlow Place
Bramalea, Ontario
L6S - 2X3

DATED December 30 1981

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled THE BIOCHEMICAL CHARACTERIZATION OF INTERLEUKIN 2 submitted by BARRY CAPLAN in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in BIOCHEMISTRY.

Supervisor

External Examiner

Date Dec 18/81

This thesis is dedicated to Thealzel, without whom it wouldn't mean as much; and to Peter, for friendship and support.

ABSTRACT

When murine spleen cells are stimulated with the mitogenic lectin Concanavalin A (Con A) they elaborate a factor, Interleukin 2 (IL2), which has a number of immunostimulatory effects in vitro. IL2 serves as a costimulator for the proliferation of Con A-stimulated thymocytes when they are cultured at low cell density. IL2 also stimulates the continuous proliferation of antigen-activated, effector T lymphocytes.

Several biochemical characteristics of IL2 were determined: (1) it has a molecular radius corresponding to globular proteins of 30,000 to 45,000 molecular weight; (2) it is an acidic molecule which at pH 7.3 elutes from DEAE-Sephacel at a salt concentration of 0.12 to 0.14 M NaCl; (3) it is separable from 'colony stimulating factor' by hydrophobic interaction chromatography on Phenyl-Sepharose; and (4) it has a heterogeneous isoelectric focusing profile, with pI values ranging from 4.6 to less than 3.4. These fractionations provide a convenient, reproducible procedure for isolating IL2 free from other lymphokines present in crude supernatants. Two activities associated with IL2 - costimulator activity and 'T cell growth' activity - copurify through all the chromatographic procedures applied.

A highly purified preparation of IL2 was used to estimate the potency of this lymphokine. IL2 is active at less than 3×10^{-12} M.

When IL2 is denatured with sodium dodecyl sulfate (SDS), quantitative recovery of activity is obtained upon removal of the SDS. A molecular weight value of 16,000 was obtained for SDS-denatured and dithiothreitol-reduced IL2 based on the results of gel filtration chromatography and glycerol gradient centrifugation. The 3 species of Interleukin 2 studied so far - rat, human and murine - can thus all exist as polypeptide chains of 15,000 - 16,000 molecular weight. The murine factor is normally isolated with a molecular weight of 31,000, suggesting that it is composed of 2 subunits.

IL2 is also produced from a variant of EL4 lymphoma cells upon stimulation with the potent tumor promotor phorbol-12-myristate-13-acetate. The IL2 produced is biochemically indistinguishable from that produced by Con A-stimulated spleen cells. The EL4 lymphoma cells produce approximately 250 fold more IL2 on a per cell basis than do Con A-stimulated spleen cells.

The defect in the ability of UV-irradiated cells to stimulate a mixed lymphocyte response is correlated with their inability to induce IL2 production. The role of IL2 in this response appears to be one of converting an antigenic signal into an immunogenic signal.

ACKNOWLEDGEMENTS

I wish to thank my supervisor, Dr. V. Paetkau for his guidance and support throughout the 4 years of research that went into this project. I am also indebted to Mr. C. Gibbs for his excellent technical assistance and for routinely performing the purification procedures detailed in this study. His assistance added a level of organization to this work that would have been lacking otherwise. I am also grateful to the other members of our research group for many interesting and helpful discussions. They provided a stimulating and pleasant environment in which to work. I am grateful to numerous members of the departments of Biochemistry and Immunology. Their helpful advice with regard to the professional and personal difficulties I encountered during my tenure as a graduate student is greatly appreciated.

I acknowledge the valuable assistance of Mr. P. D'Obrenan and Mr. R. Bradley in the preparation of this thesis. Special thanks are due Mrs. Dawn Oare. Her excellent secretarial skills and her cooperation in the preparation of this thesis are much appreciated. Finally, I acknowledge the financial assistance I received from the Department of Biochemistry, the Province of Alberta, and the Alberta Heritage Foundation for Medical Research.

ABBREVIATIONS AND DEFINITIONS

AFC:	antibody forming cell
Allogeneic:	of a different H-2 type; recognized as foreign
Ampholytes:	a series of peptide molecules of different isoelectric points; used to form pH gradients
Antigen:	a compound, usually protein or carbohydrate in nature, that can be recognized by the immune system
B cell:	antibody producing lymphocyte derived from bone marrow
BSA:	bovine serum albumin
Carrier:	an antigenic moiety recognized by helper T cells in the antibody forming cell response
Complement:	a complex series of serum proteins which lyse cells that have bound antibody
Con A:	Concanavalin A; a T cell mitogen
CTL:	cytotoxic T lymphocyte
DTT:	dithiothreitol
EDTA:	ethylenediaminetetraacetic acid
FBS:	fetal bovine serum
H-2:	major histocompatability complex in mice
Hapten:	an antigenic determinant; recognized by B lymphocytes in the antibody forming cell response
HEPES:	N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid
HP-1:	helper peak-1; same as IL 1
HS:	horse serum
Ia:	associated with the H-2 I subregion
IL1, IL2, IL3:	interleukin 1, 2 and 3
Ir:	immune response locus of the H-2 complex; a region which controls the ability of the immune system to recognize various antigenic determinants

I region:	a region of the H-2 complex
KLH:	keyhole limpet hemocyanin; an antigen
LAF:	lymphocyte activating factor; same as IL1
LPS:	bacterial lippopolysaccaride
Lyt antigens:	differentiation antigens expressed on T lymphocytes
MHC:	major histocompatability complex; genes coding for complex proteins and glycoproteins important in immune reactivity
Mitogen:	substance which stimulates a large proportion of lymphocytes to proliferate
MLR:	mixed lymphocyte response; the proliferation of T lymphocytes in response to allogeneic cells
mRNA:	messenger RNA
MW:	molecular weight
nu/nu (nude) mice:	genetically athymic mice; lack functional T cells
PAGE:	polyacrylamide gel electrophoresis
PHA:	phytohemagglutinin; a T cell mitogen
pI:	isoelectric point
PMA:	phorbol-12-myristate-13-acetate
SDS:	sodium dodecyl sulfate
SRBC:	sheep red blood cell
syngeneic:	of identical H-2 type; recognized as self
T cell:	lymphocyte which matures under thymic influence
TCGF:	T cell growth factor; same as IL2
(T,G)-A--L:	a copolymer of tyrosine and glutamic acid residues attached to a lysine backbone by alanine side chains
Thd:	thymidine
Thy 1:	a glycoprotein expressed on the surfaces of T lymphocytes
TNP:	trinitrophenol; antigen
TRF:	T cell replacing factor

Table of Contents

Chapter		Page
I.	INTRODUCTION	1
	A. THE IMMUNE SYSTEM	1
	1. Humoral and Cell-Mediated Immunity	1
	2. MHC Gene Products and T Cell Function	2
	3. T Cell-B Cell Collaboration	3
	B. THE CHARACTERIZATION OF ANTIGEN-NONSPECIFIC HELPER AND PROLIFERATIVE FACTORS	5
	1. General Considerations	5
	2. T Cell Replacing Factor	6
	a. Responses to Heterologous Erythrocytes	6
	b. Responses to Hapten-Carrier Conjugates	11
	c. Antigen-Specific Factors in the AFC Response	12
	3. Mitogenic Factors Produced By Macrophages	15
	4. Mitogenic Factors Produced By T Cells	17
	a. Thymocyte Stimulating Factor	17
	b. Costimulator	18
	c. T Cell Growth Factor	20
	d. The Interleukins	20
	C. THE ROLE OF ANTIGEN-NONSPECIFIC HELPER AND PROLIFERATIVE FACTORS IN IMMUNE REGULATION	22
	1. Interleukin 1	22
	2. Interleukin 2	24
	3. The AFC Helper Activities of IL1, IL2 and TRF	27
II.	MATERIALS AND METHODS	30
	A. ANIMALS AND TUMOR CELL LINES	30
	B. PASSAGE OF EL4 LYMPHOMA CELLS IN MICE IN THE ASCITES FORM	30
	C. TISSUE CULTURE MEDIA AND INCUBATION CONDITIONS	30
	D. PREPARATION OF SPLEEN CELLS AND IL2 PRODUCTION	31

E. IL2 PRODUCTION FROM EL4 LYMPHOMA CELLS 31
F. IL2 PRODUCTION DURING THE MIXED LEUKOCYTE REACTION 32
G. BIOLOGICAL ASSAYS FOR IL2 32
1. The Costimulator Assay 32
2. Generation and Assay of CTLs 32
3. TCGF Assay 33
H. PURIFICATION OF IL2 PRODUCED BY NORMAL SPLEEN CELLS 34
1. Ammonium Sulfate Precipitation 34
2. Sephadex G-100 Chromatography 34
3. Sephacryl S-200 Chromatography 34
4. DEAE-Sephacel Chromatography 34
I. PURIFICATION OF IL2 PRODUCED BY EL4 LYMPHOMA CELLS 35
1. Ammonium Sulfate Precipitation 35
2. Sephadex G-100 Chromatography 35
3. Phenyl-Sepharose Chromatography 35
4. DEAE-Sephacel Chromatography 35
5. Chromatofocusing 35
J. DENATURATION OF IL2 WITH SDS AND RECOVERY OF ACTIVITY 36
K. SDS-PAGE 36
L. GEL FILTRATION CHROMATOGRAPHY OF IL2 AFTER SDS DENATURATION 37
M. GLYCEROL GRADIENT CENTRIFUGATION OF IL2 AFTER SDS DENATURATION 38
N. GAMMA IRRADIATION OF CELLS 38
O. UV IRRADIATION OF CELLS 38
P. PROTEIN DETERMINATION 38
III. STUDIES ON IL2 PRODUCED FROM NORMAL MURINE SPLEEN CELLS 39
A. INTRODUCTION 39
B. RESULTS 39
1. The Costimulator Assay for IL2 39
2. The Production and Purification of IL2 40
3. Studies on SDS-Denatured IL2 42

4. The Production of IL2 During a MLR and its Abrogation by UV Irradiation 51

C. DISCUSSION 56

1. A Purification Protocol for IL2 56

2. Properties of SDS-Denatured IL2 57

The Role of IL2 in the MLR to UV-Irradiated Stimulator Cells 59

IV. STUDIES ON IL2 PRODUCED FROM EL4 LYMPHOMA CELLS 61

A. INTRODUCTION 61

B. RESULTS 62

1. TCGF Assay 62

2. Production of IL2 62

3. Cloning of the EL4 Lymphoma Cell Line 64

4. Purification of IL2 68

C. DISCUSSION 77

1. TCGF Assay 77

2. Production of IL2 from EL4 Lymphoma Cells 80

3. Purification of IL2 82

V. CONCLUDING DISCUSSION 86

REFERENCES 89

PUBLICATIONS BASED WHOLLY OR PARTLY ON THIS WORK 107

LIST OF TABLES

1. Methods used for obtaining spleen cell cultures depleted of T cells 9

2. Generation of CTLs with IL2 25

3. The mechanisms of TRF−, IL1−, and IL2− mediated helper activity for AFC responses 29

4. Purification of IL2 44

5. Recovery of IL2 after SDS denaturation 45

6. Effect of Con A and PMA stimulation on IL2 production 63

7. Production of IL2 by EL4 lymphoma cells: Effect of sera containing media and *in vitro* culture on IL2 production 66

8. IL2 production from EL4 lymphoma cells stimulated *in vitro* in medium containing either HS or FBS: Recovery of IL2 after various stages of purification 67

9. Purification of IL2 derived from EL4 lymphoma cells 74

10. Purification of IL2 by chromatofocusing: Recoveries and specific activities 76

LIST OF FIGURES

1. The costimulator assay for IL2: Comparison of ^{125}I-UdR and ^{3}H-Thd incorporation 41
2. Chromatography of fraction 3 IL2 on DEAE-Sephacel 43
3. SDS-PAGE of IL2 using the Laemmli buffer system 47
4. SDS-PAGE of IL2 using the sodium phosphate buffer system 48
5. SDS-PAGE of IL2 using the Laemmli buffer system: Experiment 2 49
6. Gel filtration chromatography of IL2 after SDS denaturation 50
7. Sedimentation velocity of IL2 after SDS denaturation 52
8. Gel filtration chromatography of IL2 produced by Con A - and MLR-stimulated spleen cells 53
9. The effect of UV irradiation of stimulator cells on IL2 production in the MLR 54
10. The effect of UV irradiation of the stimulator cells, and the addition of IL2, on the proliferative response of the MLR 55
11. Time course of IL2 production by EL4 lymphoma cells 65
12. Phenyl-Sepharose chromatography of IL2 69
13. Titration of IL2 after DEAE-Sephacel chromatography 71
14. Sephadex G-100 chromatography of fraction 5 IL2 73
15. Chromatofocusing of fraction 6 IL2 75
16. Comparison of IL2 produced by Con A-stimulated spleen cells and PMA-stimulated EL4 lymphoma cells: Chromatofocusing profiles 78
17. Gel filtration chromatography of EL4-lymphoma produced IL2 after SDS denaturation 79

I. INTRODUCTION

A. THE IMMUNE SYSTEM

1. Humoral and Cell-Mediated Immunity

The immune system, seen in its most intricate form in vertebrates, is a highly developed, adaptive, defense mechanism designed to protect the host from foreign pathogens. Historically, the effector components of the immune system were divided into humoral and cell-mediated components, based on their ability to passively transfer immunity from an immune individual to a susceptible one. In humoral immunity, transfer of immunity was achieved by using the serum of immune individuals, whereas with cell-mediated immunity, the transfer of viable cells was required.

The humoral immune response is mediated through antibodies secreted by B lymphocytes. The maturation of B lymphocytes in birds occurs in a specialized organ, the Bursa of Fabricius (therefore the designation "B" lymphocytes). In mammals, B lymphocytes are derived from the bone marrow and differentiate in secondary lymphoid organs, namely spleen, lymph nodes, and Peyer's patches.

Cell-mediated immunity is effected by two classes of T lymphocytes, cytotoxic T lymphocytes and those T lymphocytes which mediate delayed-type hypersensitivity. Cytotoxic T lymphocytes (CTLs) are an important defense mechanism against virus-infected cells and may play a role in protecting the host against tumor growth. These lymphocytes kill cells which express foreign antigens by delivering to them a 'signal' which results in their lysis. Cell-cell contact is essential for the delivery of this killing signal. The T cells responsible for delayed-type hypersensitivity, when appropriately stimulated with antigen, release chemotactic factors that attract phagocytic cells into the area of foreign antigen infiltration. Both of these classes of T lymphocytes arise in the bone marrow and mature under thymic influence (therefore the designation "T" lymphocytes). An important characteristic of T lymphocytes is the expression of a cell surface glycoprotein referred to as Thy 1 (formerly referred to as theta). The Thy 1 glycoprotein is not expressed on either B lymphocytes or macrophages.

The immune system also contains lymphocytes which exert a regulatory influence over humoral and cell-mediated responses. These regulatory lymphocytes are also T

lymphocytes (Thy 1$^+$) and they can exert either a 'helper' effect or a 'suppressor' effect on various immune responses.

Macrophages also play an important role in regulating the immune response. At one time macrophages were believed to have only one role – that of phagocytosis of foreign material. Recently it has become apparent that macrophages play a crucial role in regulating immune responses both through the presentation of antigen and through the release of immunostimulatory activities.

In addition to Thy 1 antigen, a group of cell surface antigens known as Lyt antigens are also expressed on the surfaces of T lymphocytes. These antigens have been used to distinguish between functionally diverse subsets of T lymphocytes (Cantor and Boyse 1977). In general, 'helper' T cells are Lyt 1$^+$,2$^-$,3$^-$; suppressor T lymphocytes and cytotoxic T lymphocytes are Lyt 1$^-$,2$^+$,3$^+$. The precursor T lymphocytes to both of these groups are believed to be Lyt 1$^+$,2$^+$,3$^+$ (reviewed in Cantor and Boyse 1977). Several other Lyt antigens have been described (McKenzie and Potter 1979).

2. MHC Gene Products and T Cell Function

The recognition of antigens by T lymphocytes is significantly different from the recognition of antigens by B lymphocytes. T lymphocytes have the additional requirement of recognizing a product of the major histocompatability complex (MHC) in conjunction with antigen. This restriction of antigen recognition by T lymphocytes is the basis of self / non-self discrimination.

The major histocompatability complex, designated H-2 in the mouse and HLA in humans, is a genetic locus which codes for cell surface proteins (and glycoproteins) important in immune reactivity. The H-2 complex was first recognized as being responsible for the rapid graft rejection observed when skin from one mouse was grafted onto a genetically different one, although this is not a physiologically significant function for the H-2 complex. Over the years many functions have been mapped in the H-2 locus, resulting in its division into various regions. A simplified view of the H-2 complex was recently described (Klein *et al.*, 1981), in which the H-2 complex has only one essential function – that of allowing T lymphocytes to establish self identity. The products of the H-2 locus are then divided into two classes, Class I molecules which are responsible for the development of cytotoxic reactivity, and Class II molecules which are responsible for the

development of helper and suppressor T lymphocytes. Class I molecules, the K and D proteins, are expressed on all somatic cells in an individual and are coded for by the K and D regions, respectively, of the H-2 locus. These proteins are recognized in conjunction with antigen in the response against virus-infected cells. Thus, CTLs which have been generated against virus-infected cells of one particular H-2K or H-2D phenotype would not lyse cells of a different H-2K or H-2D phenotype infected with the same virus (Zinkernagel and Doherty 1974). The CTLs must recognize both the antigen and either K or D proteins.

Class II molecules are coded for in a region of the H-2 complex designated the immune response or Ir locus (H-2 I subregion). This region codes for molecules which are expressed primarily on B cells and macrophages and are designated Ia molecules. The macrophages perform the crucial function of presenting antigen in the context of Ia molecules – a function essential for antigen recognition by both helper and suppressor T cells. Ia recognition by T cells is important, if not essential, for the development of delayed-typed hypersensitivity and for successful B cell-T cell interaction in antibody production (reviewed in Benacerraf 1981).

The distinction between Class I and Class II molecules is therefore that Class I molecules are involved in the cytolytic function of T lymphocytes, while Class II molecules are involved in the regulatory function of T lymphocytes. All T cell functions recognized so far are restricted in that they must recognize a 'self' component (a product of the H-2 complex) in addition to antigen.

3. T Cell-B Cell Collaboration

In 1966, Claman and coworkers (Claman *et al.*, 1966) demonstrated that two cell populations were required for an antibody forming cell (AFC) response to sheep red blood cells (SRBCs). They used lethally irradiated mice, whose lymphocytes are unable to proliferate. These mice serve as 'living test tubes' that allow one to investigate the roles of various injected cell populations. When either bone marrow-derived or thymus-derived cells were injected into these mice, along with antigen, the host was only able to mount a meager AFC response against the SRBCs. However, when both cell populations were injected, they synergized to give a good response, indicating, for the first time, the importance of cell-cell interaction in immune activation. Further experiments by Mitchell and Miller (1968) established that the AFCs were bone marrow-derived and that the

thymus-derived cells exerted a regulatory effect on antibody production.

An in vitro system for studying the AFC response to heterologous erythrocytes (e.g. SRBCs) was designed by Mishell and Dutton (1967). They showed that when spleen cells from normal mice were cultured in vitro for 4 to 5 days in the presence of SRBCs, the B lymphocytes recognized the SRBCs as foreign and synthesized and secreted antibody specifically directed against SRBCs. The number of responding B cells could be determined by counting the number of plaques obtained when the cultured spleen cells were mixed with SRBCs and complement. Each plaque, the result of lysis of the surrounding SRBCs, represented a single cell which had secreted antibody specific for the SRBCs. If the spleen cells were first depleted of T cells, and then challenged with SRBCs, no AFC response occurred. This result was an in vitro parallel to the results obtained by Claman *et al.*, (1966) in vivo, that T cells were required for the AFC response to SRBC.

Another system used to study AFC responses used hapten-carrier complexes. A hapten is a small antigenic moiety such as dinitrophenol which is unable to elicit an AFC response in vivo when injected in free form. However, anti-hapten antibodies are readily produced when the hapten is coupled to an immunogenic carrier, such as bovine serum albumin (BSA) or ovalbumin, before injection. The requirement for a carrier in the anti-hapten response was shown to be exerted at the level of T cells (Mitchison 1971). The T cells recognized the carrier as foreign and provided 'help ' for the B cells. The concept of 'linked recognition' was a result of studying the secondary response of B cells to hapten-carrier complexes. When mice were primed to dinitrophenol-ovalbumin and then challenged with dinitrophenol-ovalbumin or dinitrophenol-BSA, a secondary AFC response was obtained only with the homologous carrier (dinitrophenol-ovalbumin). The injection of dinitrophenol-BSA and free ovalbumin into dinitrophenol-ovalbumin primed mice did not result in a secondary response, indicating that the B cells recognizing dinitrophenol and the T cells recognizing ovalbumin had to be in close proximity. A secondary response to dinitrophenol-BSA could be obtained in dinitrophenol-ovalbumin primed mice if these mice were also primed with free BSA. In the presence of the appropriate hapten-carrier conjugate (dinitrophenol-BSA), the T cells primed to BSA interacted (provided help) to B cells primed to dinitrophenol.

B. THE CHARACTERIZATION OF ANTIGEN-NONSPECIFIC HELPER AND PROLIFERATIVE FACTORS

1. General Considerations

The observation that T lymphocytes and macrophages exert a regulatory influence over both B lymphocytes and other T lymphocytes has led to considerable interest in determining the mechanism by which such regulation takes place. Two possible regulatory interactions can be envisaged – regulation which is mediated through cell–cell contact or regulation mediated by soluble effector molecules. Both T cells and macrophages release soluble effectors which influence T cell function, B cell function, and macrophage function (reviewed in Waksman and Namba 1976). These soluble effectors may be stimulatory or inhibitory and they may exert their function in an H–2 restricted or unrestricted manner (H–2 restricted factors would only influence cells which are syngeneic to the cells which produce the factors). Finally, these soluble effectors may function in either an antigen–specific or nonspecific manner. Antigen–nonspecific factors stimulate an immune response to several completely different antigens, and their activity is independent of the method used to produce them.

There is considerable confusion as to the role of these various factors in immune regulation. It has become apparent over the last several years that the crude supernatants of lymphoid cell cultures contain a variety of both stimulatory and inhibitory factors. In addition, several of the assay systems used to characterize these factors respond to 2 or more different factors. Thus an understanding of the role of soluble effectors in the immune response requires the study of biochemically well characterized factors in clearly defined assay systems.

Among the first reports of soluble factors capable of stimulating immune responses were those of Kasakura and Lowenstein (1965) and Gordon and Maclean (1965). These investigators observed that cell–free media from cultures of human leukocytes stimulated other human leukocytes to proliferate. Over the next several years, a wide variety of soluble factors which had an influence on the immune system were described. The remainder of this discussion will consider those factors that are antigen–nonspecific and which either stimulate proliferation or supply 'help' in the immune response. These factors could be divided into what at first appeared to be three distinct groups. The first

group of factors stimulated the proliferation of leukocytes. The factors described by Kasakura and Lowenstein (1965) and Gordon and Maclean (1965) fit into this category. As well, Janis and Bach (1970) described a factor, also obtained from human leukocytes cultured in vitro, which in addition to stimulating proliferation had 'potentiating' activity. The potentiating effect referred to the factor's ability to synergize with antigen in stimulating proliferation. The response observed was greater than the sum seen with antigen alone and with factor alone. A potentiating factor for mouse thymocyte responses was reported by Gery *et al.* (1971).

The second group of nonspecific factors replaced the requirement for macrophages in several different immune responses. Bach *et al.* (1970) showed that cultured macrophages produced a factor which restored the proliferative response of T cells in macrophage-depleted cultures. Another macrophage-produced factor replaced macrophages in the in vitro AFC response to SRBCs (Hoffmann and Dutton 1971).

The third group of nonspecific factors replaced T cells in the AFC response to SRBCs. This activity was first described by Dutton *et al.* (1971) and subsequently by Schimpl and Wecker (1972), and others (Doria *et al.* 1972; Feldman and Basten 1972; Gorczynski *et al.* 1972). These three groups of factors, among others, were lymphokines: non-antibody mediators of cellular immunity generated by lymphocyte activation (Dumonde *et al.* 1969).

The following discussion will consider the events which have led up to our present understanding of the role of nonspecific helper and proliferative factors in the immune response. In addition, antigen-specific factors active in AFC responses will also be discussed. In general, the events described will refer to the study of the murine factors both because they are the best characterized and because the results presented in this thesis involve the murine system exclusively.

2. T Cell Replacing Factor

a. Responses to Heterologous Erythrocytes

The experiments by Claman and coworkers (Claman *et al.* 1966) and Mitchell and Miller (1968) demonstrated that T cells played an important role in B cell activation in vivo. Experiments using the Mishell-Dutton culture system confirmed in vitro the requirement for T cells in the AFC response to SRBCs. In 1971, Dutton and coworkers (Dutton *et al.*

1971) demonstrated that the cell-free supernatants from suitably activated T cells could replace the requirement for T cells in the AFC response to SRBCs. Shortly thereafter, Schimpl and Wecker (1972) proposed the term T Cell Replacing Factor (TRF) for an activity similar to the one described by Dutton. Several other investigators also described TRF activity (Doria *et al*. 1972; Feldmann and Basten 1972; Gorczynski *et al*. 1972; Sjoberg *et al*. 1972). These reports, that a nonspecific soluble effector could replace the requirement for T cells in the AFC response in vitro, suggested that one mechanism of T cell-B cell collaboration was through the release of a soluble mediator.

TRF activity is obtained in cell-free supernatants when cultures of T cells are appropriately stimulated with antigen. It is generally observed however, that the number of virgin lymphocytes (those that have not encountered antigen) that respond to any one particular antigen is severely limited. This is expected on the basis of the Clonal Selection Theory formulated by Burnet (1959); each lymphocyte has receptors for one antigen only. In response to that antigen the lymphocyte proliferates, producing progeny with receptors specific for the stimulating antigen (clonal selection). In the case of B lymphocytes, the proliferating cells produce and secrete large quantities of antibody with specificity towards the antigen. To efficiently generate TRF it is therefore necessary to use culture conditions in which a large number of T cells can be stimulated. One of three culture conditions is commonly used. In the first, activated T cells are generated by injecting thymocytes into lethally irradiated, syngeneic recipients which are then immunized with a particular antigen (reviewed in Katz 1977, Chapter 1). The principle is that a substantial proportion of the spleen cells obtained from these mice, after about 1 week, are primed T cells derived from the antigen-stimulated thymocytes. As a result a much larger response occurs when these cells are stimulated with the priming antigen than would be seen using unprimed cells.

Another method commonly used to efficiently generate TRF is the mixed leukocyte reaction (MLR). First described by Bach and Hirshlorn (1964), and Bain *et al*. (1964), the MLR is the proliferative response seen when leukocytes from two different individuals are cultured together. This proliferation is a result of T lymphocytes recognizing as foreign the histocompatability antigens present on the stimulator cells. The MLR can be made uni-directional by treating one set of cells with mitomycin C or x-irradiation, thereby

inhibiting their proliferation. In comparison to antigenic stimulation, a much higher frequency of unprimed T cells recognize and respond to foreign histocompatability antigens. The biological significance of this increased frequency may be related to the double requirement for antigen and self recognition of T cell responses.

The third method commonly used to produce TRF is polyclonal activation by mitogens. Mitogens are agents which stimulate lymphocyte proliferation in a manner that does not involve antigen receptors on the lymphocytes. The stimulation requires binding of the mitogen to various cell surface components, in particular the charbohydrate moieties of various glycoproteins (Greaves and Janossy 1972) . Two commonly used mitogens are phytohemagglutinin (PHA) and Concanavalin A (Con A) – plant lectins which specifically stimulate T lymphocytes. B lymphocytes of various specificities can be stimulated to proliferation and antibody secretion by lipopolysaccharide (LPS), a mitogen obtained from Escherichia coli. Pokeweed mitogen stimulates both B and T cells. The response to mitogens is often referred to as polyclonal because cells with many different specificities are stimulated to proliferate.

The preliminary studies on TRF demonstrated that it was produced in all three culture systems described above. Cell–free supernatants containing TRF activity have most commonly been obtained by MLR stimulation of T cells (Schimpl and Wecker 1972; Dutton *et al*. 1971; Sjoberg *et al*. 1972). As well, TRF activity is produced by Con A–stimulated T cells (Wecker *et al*. 1975; Waldmann *et al*. 1976), T cells stimulated by SRBCs (Gorczynski *et al*. 1972; Doria *et al*. 1972), and activated T cells stimulated with the priming antigen (Waldmann and Munro 1973; Gisler *et al*. 1973). Although T cells are clearly implicated in TRF production, the heterogeneity of the starting spleen cell population does not allow conclusive proof that the T cells synthesize TRF. In general, it is easier to determine which cells are important in producing a factor than it is to determine which cells synthesize it. Clearly T cells, in particular Lyt 1^+ helper T cells (Pickel *et al*. 1976), are involved in TRF production, but a requirement for macrophages has also been shown (Sjoberg *et al*. 1972; Waldmann and Munro 1973).

The standard assay for TRF measures its ability to replace T cells in the primary immune response to SRBCs. Several methods were commonly used to obtain spleen cells deficient in T cells to use in this assay. These methods are described in Table 1.

TABLE 1

METHODS USED FOR OBTAINING SPLEEN CELL CULTURES DEPLETED OF T CELLS

Spleen cells obtained from:	Basis of the T Cell Deficiency	References for use as a TRF Assay
1. normal mice. The spleen cells are treated with Thy-1 antisera (+complement) *in vitro*.	Functionally mature T cells express the cell surface antigen Thy-1. The treatment of spleen cells with Thy-1 antisera (+complement) results in the lysis of essentially all functional T cells.	Dutton *et al.* (1971) Gorczynski *et al.* (1972, 1973a,b) Schimpl and Wecker (1972) Sjoberg *et al.* (1972)
2. nu^+/nu^+ (nude) mice.	The differentiation of functional T cells requires a thymus. Nude mice are genetically athymic.	Askonas *et al.* (1974) Wecker *et al.* (1975)
3. neonatally thymectomized mice.	No functional T cells can mature in neonatally thymectomized mice due to the absence of the thymus.	Doria *et al.* (1972)
4. adult thymectomized, lethally-irradiated, and bone marrow-reconstituted mice.	The bone marrow cells used to repopulate the lethally-irradiated mice are devoid of functional T cells and in the absence of a thymus cannot mature into functional T cells.	Waldmann and Munro (1973, 1974) Gorczynski *et al.* (1973a) Hunter and Kettman (1974)
5. mice treated *in vivo* with rabbit anti-mouse thymocyte serum (+complement). The spleen cells are then treated *in vitro* with anti-T serum (+complement).	In addition to removing functional T cells, this procedure also removes immature thymocytes which can differentiate into functional T cells.	Harwell *et al.* (1976)

The simplest explanation for the mechanism of TRF activity is that TRF stimulates B cells directly to produce antibody. The first experiments of Schimpl and Wecker (1972) demonstrated that TRF worked best when added 2 days after the initiation of culture. This suggested that at least 2 signals were involved in B cell activation, one which worked early (antigen) and the other which worked late (TRF). Experiments by Hunig *et al*. (1974), using an autoradiographic analysis of plaque forming cells, showed that TRF had no effect on the proliferation of AFCs in culture. This observation was extended by Dutton (1975) when he showed that the cells which proliferated in response to antigenic stimulation were the cells which were subsequently responsive to T cell help. The interpretation of these results, and others (Askonas *et al*. 1974), was that antigen was providing signal 1 for B cell activation (proliferation) and that TRF was providing signal 2 (differentiation).

A slightly different mechanism for TRF activity was proposed by Hunter and Kettmann (1974) and Waldmann *et al*. (1976). They provided evidence that TRF stimulated both proliferation and differentiation of AFCs. By using a limiting dilution analysis, these investigators demonstrated that both the frequency of B cells able to respond to antigen and the number of AFCs obtained from each responding B cell were increased by the addition of TRF-containing supernatants. These effects were observed when TRF was added on day 0, in contrast to the results obtained by Schimpl and Wecker (1972). The discrepancy between these 2 different mechanisms of TRF action is partly related to the use of crude supernatant material. It has recently been shown that these crude supernatants can contain lymphokines other than TRF and that these lymphokines can stimulate B cell proliferation (see Section C3).

Up to this stage, there was very little attempt to charaterize the molecules involved in TRF activity (for an exception see Watson 1973). There was some suggestion that the TRF produced by MLR stimulation was different from that produced by activated T cells (Waldmann and Munro 1974). However, Harwell *et al*. (1976) showed that TRF produced by MLR stimulation, Con A stimulation and activated T cells stimulated with the priming antigen, were indistinguishable in terms of molecular size. All three eluted from a Sephadex G-200 column with standard proteins of 30-40,000 molecular weight. In addition, the dose response curves for TRF activity were similar in all 3 cases.

Further attempts at characterizing TRF biochemically were the studies of Schimpl and Wecker and coworkers (Muller *et al*. 1978; Hubner *et al*. 1978). Using TRF which had been partially purified through several chromatographic procedures, it was shown that TRF was a protein, based on its sensitivity to heat and proteases, and that it probably contained carbohydrate moieties. No Ia or H-2 related components were apparent. Different forms of TRF were shown to have apparent molecular weights of 45,000, 30-35,000 and 25,000. These investigators also determined that TRF activity was detectable at less than 10^{-10} M. Based on the amount of TRF activity present in crude supernatants, it was estimated that less than microgram amounts of TRF protein were present in these supernatants. Total protein, on the other hand, was in the range of milligram amounts, thus indicating the difficulty in purifying TRF to homogeneity. The nonspecific nature of TRF precluded the use of any specific affinity resins, leaving only standard biochemical techniques for purification.

The culture conditions used to produce TRF were subsequently shown to produce other lymphokines. These lymphokines were active in stimulating (and suppressing) various immune responses involving T cells, B cells, and macrophages (see sections B4 and B5). An understanding of the role of TRF in B cell activation thus carried two requirements: (1) the isolation of TRF material free of other immunoregulatory factors and (2), the development of assay systems specific for TRF and not responsive to other lymphokines. These two objectives have been partly realized and will be discussed in detail later (see Section C3). It seems likely that TRF does indeed stimulate the last stage of differentiation in the AFC response to antigenic stimulation, as initially proposed by Wecker *et al*. (1975).

b. Responses to Hapten-Carrier Conjugates

T cell-B cell collaboration is not limited to the primary immune response of B cells to heterologous erythrocytes. Several laboratories have studied the T cell requirement for an AFC response to soluble hapten-carrier conjugates (Gisler *et al*. 1973; Marrack (Hunter) and Kappler 1975; Harwell *et al*. 1976; North *et al*. 1977; Hunig *et al*. 1977a,b; Waldmann and Munro 1974). In contrast to the results obtained in generating an AFC response to SRBCs, the requirement for T cells in the response to soluble hapten-carrier conjugates could not be replaced by non-specific TRF (Gisler *et al*. 1973; Waldmann 1975). Gisler *et al*. (1973) generated TRF activity from 3 separate groups of activated T cells - those

primed and stimulated with horse red blood cells, SRBCs, and keyhole limpet hemocyanin (KLH). All three TRFs stimulated the AFC response of nude spleen cells to either horse red blood cells or SRBCs. However, only the TRF produced from activated T cells primed and stimulated with KLH was active in stimulating the AFC response to dinitrophenol–KLH. Thus, the supernatant from activated T cells primed and stimulated with KLH contained both an antigen–specific component for KLH and an antigen–nonspecific component active in the response to heterologous erythrocytes.

In a series of experiments using hapten primed B cells, Marrack and Kappler and coworkers (Marrack (Hunter) and Kappler 1975; Harwell *et al*. 1976; Keller *et al*. 1980) were able to show that the AFC response to trinitrophenol–KLH required two types of helper T cells. The first functioned in an antigen–specific manner in the initiation of proliferation of B cells. The second type of helper T cell acted in an antigen nonspecific manner and appeared to promote differentiation after the initial induction of proliferation. This second type of helper T cell could be replaced by TRF, and was the only requirement for generating an AFC response to heterologous erythrocytes.

In contrast to these results, several investigators reported that antigen–nonspecific factors were sufficient in stimulating the AFC response to protein antigens (Hunig *et al*. 1977a,b; North *et al*. 1977). Thus North *et al*. (1977) demonstrated that MLR–produced TRF stimulated the AFC response of dinitrophenol–primed B cells to dinitrophenol–KLH.

The discrepancy between the different requirements for B cell activation in response to hapten–carrier conjugates is not easily resolved. It is quite likely that the particular maturational state of the B cells used in the AFC response dictates the precise requirement for stimulation. The slight variations used by different investigators in obtaining primed B cells could therefore result in significantly different requirements for activation.

c. Antigen–Specific Factors in the AFC Response

Antigen–specific factors are products of helper and suppressor T cells that regulate the response of B lymphocytes in an antigen–specific manner. Both antigen–specific helper factors and antigen–specific suppressor factors have been described. Although these two groups of factors exert opposite effects, they do have several important

features in common. These features, listed below, are reviewed by Taussig (1980), and Taussig *et al.* (1980). Of particular interest are the observations that antigen-specific factors: (1) bind the antigen that they are specific for; (2) lack the constant region of immunoglobulins but carry the variable determinants of immunoglobulin heavy chain; and (3) contain determinants coded for by the H-2 I subregion of the major histocompatability complex. These three observations have led to considerable interest in these factors both as a mechanism of cell - cell interaction and as the possible (and elusive) antigen receptor on T cells.

Antigen-specific helper factors for (T,G)-A--L (Taussig 1974, Howie *et al.* 1979), GAT (Howie *et al.* 1979), KLH (Tokuhisa *et al.* 1978) and rabbit gamma-globulin, human gamma-globulin, BSA and chicken red blood cells (Shiozawa *et al.* 1977, 1980) have been reported. [(T,G)-A--L is a copolymer of tyrosine and glutamic acid residues attached to a lysine backbone by alanine side chains. GAT is a copolymer of glutamic acid, alanine and tyrosine]. The KLH-specific helper factor requires T cells for its activity (Tokukisa *et al.* 1978). The other factors do apparently function in the absence of T cells. This suggests that antigen-specific helper factors can work through two different mechanisms - the stimulation of helper T cells which then provide help for the B cells, and, the stimulation of B cells directly. The carrier-specific helper factor for chicken red blood cells described by Shiozawa *et al.* was recently shown to work only in conjunction with adherent cells, or their nonspecific soluble products (Shiozawa *et al.* 1980).

Recently, several T cell hybridomas have been reported which secrete antigen-specific helper factors (Hiromatsu *et al.* 1981; Eshhar *et al.* 1980; Lonai *et al.* 1981a). A T cell hybridoma is the fusion product between a normal T cell and one which is neoplastic. A successful partnership results in a T cell hybrid which retains both the immunological function of the normal T cell and the immortality of the neoplastic one. The subsequent cloning and growth of a T cell hybridoma provides a homogeneous source of T cells and T cell products. These hybridomas should therefore prove to be an excellent source of antigen-specific helper factors for biochemical characterization.

Antigen-specific suppressor factors have been reported for KLH (Tada *et al.* 1979, 1980; Kontiainen and Feldmann 1978), GAT and GT (Pierce *et al.* 1979; Germain and Benacerraf 1978), and SRBCs (Taussig *et al.* 1979c). These factors are active in either: (1)

stimulating the generation of suppressor T cells; (2) inhibiting the action of helper T cells; or (3) suppressing the response of B cells directly (reviewed in Taussig *et al.* 1979c).

Several hybridomas have been shown to produce antigen-specific suppressor factors (Kontiainen *et al.* 1978; Taniguchi *et al.* 1979, 1980; Taussig *et al.* 1979a,b). The advantage of having a hybridoma which produces antigen specific suppressor factor was exemplified in the recent studies of Tada and coworkers (Taniguchi *et al.* 1979, 1980). The antigen-specific suppressor factor for KLH produced from a hybridoma was shown to be composed of two separate chains - one responsible for binding antigen and presumably containing variable determinants of immunoglobulin heavy chain, and the other composed of an H-2 I subregion determinant. These two chains are synthesized independently in the cytoplasm and secreted in an associated form. This was the first demonstration that an antigen-specific factor was composed of two separate chains coded for separately in the genome.

One of the most intriguing observations related to antigen-specific suppressor factors was the demonstration by Taniguchi and Tokuhisa (1980) that the antigen-specific suppressor factor for KLH stimulated the generation of suppressor cells which were able to mediate suppression in an antigen-nonspecific and H-2 unrestricted manner. This observation relates both specific and nonspecific mechanisms of suppression. It demonstrates that following induction which is antigen-specific and H-2 restricted, suppression can be effected by nonspecific mediators. The retention of specificity *in vivo* is most probably achieved by complex interactions involving macrophages, B cells and the nonspecific suppressor cells such that 'bystander' cells are not suppressed.

Another factor implicated in triggering B cell responses is allogeneic effect factor. Allogenic effect factor is produced in a MLR which uses as responders T cells activated *in vivo* by injecting them into allogeneic hosts. These *in vivo*-activated T cells are then stimulated with irradiated spleen cells of host origin. It has recently been shown that allogeneic effect factor produced by this mechanism has two components (Delovitch *et al.* 1981; reviewed in Delovitch and Phillips, 1981). Component I, of molecular weight (MW) 50-70,000, is an antigen-specific factor for allogeneic Ia antigens. It has been proposed that component I stimulates B cells by interacting with the Ia antigens on their surfaces, thus providing signal 2 for activation. Signal 1 in this situation is antigen. Component II is a

nonspecific mitogenic factor for T cells indistinguishable from Interleukin 2, to be described later).

3. Mitogenic Factors Produced By Macrophages

Lymphocyte activating factor (LAF) was first described as a factor which could synergize with the T cell mitogen PHA in stimulating thymocytes to proliferate (Gery *et al.* 1971). The combination of LAF-containing supernatants and PHA stimulated thymocyte proliferation to an extent greater than the sum seen with PHA alone and with LAF alone (the synergistic effect). LAF was also shown to be mitogenic in itself.

LAF was produced by unstimulated human peripheral blood leukocytes. However, more LAF was generated when the peripheral blood leukocytes were stimulated with PHA or LPS (Gery *et al.* 1971). LAF was produced by human, rabbit, rat, and mouse lymphoid cells upon stimulation with LPS (Gery *et al.* 1972). PHA and Con A stimulation of lymphoid cells also elicited LAF in some species. Adherent cells were responsible for producing LAF although a requirement for T cells could not be eliminated (Gery and Waksman 1972). LAF was reported to have a MW of approximately 15,000 as determined by gel filtration (Gery and Handschumacker 1974).

Several investigators reported the presence of other factors in LAF containing supernatants. Calderon and Unanue (1975) reported that cultured peritoneal exudate cells (cells obtained by a lavage of the peritoneal cavity of mice) produced LAF and a low molecular weight factor which inhibited the proliferative response of mouse thymocytes. This observation resolved some of the confusion surrounding the different levels of LAF present under various conditions. The stimulation seen was a result of the relative amounts of these two factors present in the sample assayed.

A significant advance was the discovery that several macrophage-like murine tumor cell lines could synthesize LAF (Lachman *et al.* 1977a). The cell lines P388D_1, J774.1, WEHI3, and PU5-1.8 all produced LAF activity when stimulated with LPS. Several different molecular weight forms of LAF were generated from these tumor cell lines. In addition to the 15,000 MW form (Mizel and Rosentreich 1979), LAF activity was observed with MWs of 50-60,000 and 75-80,000 (Lachman *et al.* 1977a). Later studies by Mizel and Rosenstreich (1979) suggested that the high MW forms of LAF represented intracellular precursors for the 15,000 MW, secreted form. These experiments proved

that LAF could be elicited directly from macrophages and that T cells were not essential for LAF production.

Several of the tumor cell lines synthesized LAF in response to stimuli other than LPS. $P388D_1$ cells produced LAF in response to phorbol-12-myristate-13-acetate (PMA) or activated T cells (Mizel *et al.* 1978a,c). The J774.1 cell line was shown to produce LAF in response to 8-Br-cyclic AMP (Okada *et al.* 1978).

These tumor cell lines are an excellent source of LAF for biochemical characterization. LAF with a MW of 16,000 and which exibited charge heterogeneity as determined by ion-exchange chromatography was produced by $P388D_1$ cells either unstimulated or stimulated with LPS or activated T cells (Mizel *et al.* 1978b). The LAF produced by $P388D_1$ cells stimulated with PMA was shown to be composed of a single polypeptide chain of 12-16,000 molecular weight as determined by SDS polyacrylamide gel electrophoresis (PAGE) (Mizel 1979). The behaviour of LAF on SDS gels was based on the activity eluted from the gel and assayed after removal of SDS.

The properties of LAF from murine peritoneal exudate cells stimulated with LPS were most thoroughly studied by Economou and Shin (1978). It was shown to have an isoelectric point of 4.8 and a MW of 18,000. The MW was calculated using the Svedberg equation and took into consideration experimentally derived values for the molecular radius, the buoyant density and the sedimentation coefficient of LAF.

Studies on human LAF showed it to have a MW of 13,000 (similar to mouse LAF) and an isoelectric point of 6.8 (higher than the pI of 4.8 for mouse LAF) (Blyden and Handschumacher 1977; Lachman *et al.* 1977b, 1978). Lachman *et al.* (1977b) were able to purify human LAF by greater than 16,000 fold and concluded that LAF was detectable in sub-microgram amounts.

In adddition to LAF, human peripheral blood leukocytes were shown to elaborate factors which could replace T cells in the AFC response to SRBCs (Farrar, J.J. and Fuller-Bonar 1976; Farrar, J.J. *et al.* 1977). One of these helper factors, helper peak-1, was shown to be similar, if not identical to LAF (Koopman *et al.* 1977, 1978). Both the thymocyte proliferative activity of LAF and the helper activity characteristic of helper peak-1 were shown to behave similarly on several chromatographic fractionations including gel filtration, ion-exchange chromatography on DEAE-sepharose and

CM-sepharose, and PAGE under non-denaturing conditions.

Somewhat similar studies by Wood (1979a,b and references contained within) demonstrated the similarity between LAF and 'B cell activating factor'.

In the mouse system, similar events took place. Hoffmann *et al.* (1979) described a macrophage-produced factor from LPS-stimulated peritoneal cells which stimulated the AFC response of nude mouse spleen cells to antigens bound to heterologous erythrocytes. This factor was referred to as B cell differentiation factor and later as T cell replacing factor - macrophage (Hoffmann and Watson 1979). It was shown to have an approximate MW of 15,000 and was proposed to be similar, if not identical, to LAF (Hoffmann and Watson 1979).

4. Mitogenic Factors Produced By T Cells

a. Thymocyte Stimulating Factor

In 1975, a thymocyte stimulating factor which was apparently not produced by macrophages was reported (DiSabato *et al.* 1975). This 'thymocyte stimulating factor' was produced by PHA- stimulated spleen cells and it conferred upon thymocytes the ability to respond to PHA. 'Thymocyte stimulating factor' was different from LAF by several criteria. As already mentioned, macrophages were not required for its production. In addition, 'thymocyte stimulating factor' was not mitogenic in itself, it required the presence of PHA for mitogenic activity (DiSabato *et al.* 1975). Most importantly, however, 'thymocyte stimulating factor' was shown to have a molecular weight (as determined by gel filtration) of 30-32,000 (Chen and DiSabato 1976).

In most of the early literature on these and other thymocyte stimulating factors a distinction was made between their mitogenic and synergistic properties. Some of these factors, notably LAF, were both mitogenic and synergistic. Other thymocyte stimulating factors were only synergistic. The synergistic response, however, is usually many fold greater than the mitogenic response. Therefore, at a particular dilution a factor might appear to be synergistic but not mitogenic. Complicating this problem is the fact that the assay systems used by various investigators were significantly different in terms of cell number, concentration or presence of mitogen, incubation time, and labelling time (for the incorporation of tritiated thymidine). The difference between mitogenic and synergistic activity is therefore most probably artifactual (see also Paetkau 1981).

'Thymocyte stimulating factor' was suggested to be a factor which stimulated thymocyte maturation (Chen and DiSabato 1976, 1977). The majority of thymocytes are generally accepted to be functionally immature T cells, which must undergo some form of differentiation within the thymus before leaving it as mature T cells. Chen and DiSabato (1976, 1977) observed that 'thymocyte stimulating factor' allowed thymocytes to proliferate in a MLR, to respond to Con A stimuation, and to participate in a graft-versus-host response - functions characteristic of mature T cells. Beller and Unanue (1977) demonstrated that a similar factor (30-40,000 MW) also allowed thymocytes to respond in an MLR, and in addition, increased the amount of surface H-2, and decreased the susceptibility of thymocytes to anti-thymus-leukemia antigen treatment - further characteristics of mature T cells.

In addition to its production by PHA-stimulated spleen cells, 'thymocyte stimulating factor' activity was also generated during a MLR and by KLH-primed spleen cells in response to KLH. 'Thymocyte stimulating factor' was not produced by nude mouse spleen cells (Chen and DiSabato 1976; DiSabato *et al*. 1978). Furthermore, 'thymocyte stimulating factor' was shown to require Thy-1 positive T cells, RNA and protein synthesis, but not DNA synthesis for its production (DiSabato *et al*. 1978).

b. Costimulator

A factor which allowed thymocytes to respond to PHA was described by our group and referred to as costimulator (Paetkau *et al*. 1976; Mills *et al*. 1976; Shaw *et al*. 1978a,b). Its biochemical characterization is the subject of this thesis. Several characteristics of costimulator will, however, be described here so that its relationship to other soluble effectors can be appreciated.

Costimulator is produced by Con A-stimulated spleen cells and it allows thymocytes to proliferate in response to Con A and PHA; it is therefore a costimulator for thymocyte mitogenesis (Paetkau *et al*. 1976; Mills *et al*. 1976). Normally, costimulator requires both macrophages and T cells (Thy 1^+ cells) for its production (Paetkau *et al*. 1976; Shaw *et al*. 1978a), and its activity is directed at T cells. Mouse costimulator is a protein (trypsin sensitive) or glycoprotein with a molecular weight of approximately 31,000 as determined using the Svedberg equation (Shaw *et al*. 1978b).

Several aspects of the earlier work deserve mention. The first is that a quantitative assay for costimulator was devised (Shaw *et al*. 1978a,b). This was achieved by culturing thymocytes at a cell density of less than 1 x 10^6 cells/ml. At this concentration, the thymocytes are no longer responsive to stimulation with Con A and their proliferation is totally dependent on the amount of costimulator added to the culture (Shaw *et al*. 1978b). This assay facilitated a quantitative estimation of costimulator and allowed a comparison of its potency in stimulating various immune responses.

Another important aspect of the work on costimulator was the demonstration that it was active in stimulating several immune responses in vitro. Costimulator stimulated the proliferative response of thymocytes to both Con A (the standard assay system) and PHA. More importantly, CTLs specific for alloantigen could be generated from thymocyte precursors in the presence of costimulator (Shaw *et al*. 1978a).

As mentioned previously, CTLs are lymphocytes which mediate the lysis of cells bearing foreign antigens. In 1975, Cantor and Boyse (1975) demonstrated that two subclasses of T cells were involved in the generation of CTLs. One subclass mediated the effector lytic function and the other amplified the development of killer activity. The cells which mediated the amplifier effect were Lyt 1^+ helper T cells. Probably the most direct evidence for the involvement of helper T cells in generating a CTL response derived from the demonstration that thymocytes are poor responders in generating CTLs because they lack differentiated helper cells. Pilarski (1977) showed that when helper cells obtained from peripheral lymphoid organs were added to thymocyte cultures, CTLs were efficiently generated. Thus, thymocytes contain the precursors which can mediate CTL effector function, but they lack the amplifying effect of helper T cells. The observation that costimulator allowed thymocytes to develop cytotoxic reactivity suggested that it overcame the requirement for helper T cells (Shaw *et al*. 1978a, 1980).

Costimulator also stimulated the CTL response of spleen cells to glutaraldehyde-fixed stimulator cells (Shaw *et al*. 1978a). Material which had been purified by gel filtration and isoelectric focusing (and detectable at less than 10^{-10} M) was active in all of the responses described above. This suggested that these responses were induced by similar, if not identical, factors.

These experiments led to the proposal that costimulator was a second signal for the stimulation of various T cell responses (Mills *et al*. 1976; Shaw *et al*. 1978a), in analogy to T cell replacing factor being the second signal for B cell responses. Antigen or mitogen functions as the first signal in both cases.

c. T Cell Growth Factor

In 1977, Gillis and Smith (1977) demonstrated that Con A–stimulated spleen cells elaborated a factor which allowed T lymphocytes to grow continually in culture. Morgan *et al*. (1976) demonstrated that a factor (in this case produced by PHA–stimulated human leukocytes) stimulated the continuous proliferation of human T lymphocytes in culture. Gillis and Smith (1977) extended these observations to the murine system and showed that cytotoxic T lymphocytes could be maintained continuously in culture in the presence of this factor. The factor responsible for this activity was called T cell growth factor (TCGF). TCGF was subsequently shown to be produced by Thy 1$^+$ T cells in response to Con A stimulation in both the murine and rat systems. TCGF was also produced during a MLR, and its activity was removed by proliferating T cells (Gillis *et al*. 1978b).

A quantitative microassay for TCGF activity was designed by Gillis *et al*. (1978b) which made use of the CTL lines which had been grown continually in culture for greater than 1 year. These CTL lines were totally dependent on TCGF for their continued proliferation – cell death occurred within 24 hours after its removal (Gillis *et al*. 1978b).

d. The Interleukins

By 1978, a large number of factors was known to be present in cultures of Con A– or MLR–stimulated spleen cells. As mentioned previously, TRF (Section B2), 'thymocyte stimulating factor' (Section B4a) costimulator (Section B4b) and TCGF (Section B4c) were all produced in spleen cell cultures by either Con A stimulation, MLR stimulation, or both. Also present in these supernatants were factors which: (1) replaced the requirement for helper T cells in the generation of CTLs from spleen cell cultures (Plate 1976, 1977); (ii) allowed spleen cells to produce CTLs in response to non–stimulating tumor cells (Talmage *et al*. 1977); (iii) allowed thymocytes to serve as stimulators in the generation of CTLs by spleen cells (Finke *et al*. 1977) and (iv) stimulated the secondary response of CTLs to allo–antigens (the 'secondary cytotoxic T cell–inducing factor') (Wagner and Rollinghoff 1978).

In 1978, Farrar and co-workers (Farrar, J.J. *et al*. 1978) proposed that a single mediator was responsible for both T cell and B cell activation. It was demonstrated that 3 activities present in Con A supernatants co-purified through ammonium sulfate precipitation and several chromatographic procedures (gel filtration, hydroxylapatite and Phenyl-Sephaose chromatography). The factors responsible for these activities were called thymocyte mitogenic factor (stimulated the proliferation of thymocytes), killer cell helper factor (stimulated the generation of CTLs from thymocyte precursors), and T cell replacing factor (stimulated the AFC response of nude spleen cells). These experiments also suggested that more than one T cell replacing factor was present in supernatants from Con A-stimulated spleen cells. One T cell replacing factor was associated with thymocyte mitogenic activity, and the other was the late-acting (active when added on day 2 of the 5 day assay) TRF as described by Schimpl and Wecker (1972).

The conclusion that one factor was responsible for all 3 activities described above was also reached by Watson *et al*. (1979b) who showed that the material responsible for the 3 activities co-purified through ion-exchange chromatography and isoelectric focusing, as well as salt precipitation and gel filtration. A further dimension of this paper was the demonstration that these factors acted in the same concentration range supporting the contention that the same entity was responsible for them. TCGF activity also co-purified with costimulator activity, killer helper factor activity and T cell replacing factor activity (Watson *et al*. 1979b).

These observations, and the equivalent ones regarding the mitogenic factors produced by macrophages (Section B3) led the participants of the Second International Lymphokine Workshop in Ermatingen, Switzerland to propose the terms Interleukin 1 (IL 1) and Interleukin 2 (IL2) for the macrophage- and T cell- produced proliferation and helper factors respectively (Aarden *et al*. 1979). The term 'Interleukin' indicates that these factors are a mechanism of communication between leukocytes.

Interleukin 1 is the macrophage produced factor previously referred to as LAF, helper peak-1, TRF-macrophage, B cell activating factor and B cell differentiation factor (for references see Aarden *et al*. 1979, and Section B3). IL 1 has a molecular weight of 12-16,000, and an isoelectric point in the range of 4.5-5.5 for mouse and 6.5-7.5 for human. IL 1 activity is antigen-nonspecific, H-2 unrestricted and species unrestricted.

Interleukin 2 is a factor produced by T cells and previously referred to as 'thymocyte stimulating factor', costimulator, TCGF, thymocyte mitogenic factor and killer cell helper factor. Murine IL2 has a molecular weight of 30–35,000 and an isoelectric point of 3.5–5.5. IL2 activity is antigen–nonspecific and H–2 unrestricted. Unlike IL 1 activity, IL2 activity is partly species restricted.

An important distinction between IL 1 and IL2 is that only IL2 has the TCGF activity described by Gillis and Smith (1977). IL 1 does not stimulate the continuous proliferation of T cells. The importance of this assay cannot be overstated. The cells used for the TCGF assay can be derived from a single cell (i.e., cloned). As a result, these cells cannot be influenced by secondary effects contributed by 'bystander' cells as can all of the other assays described so far. This assay is only responsive to factors which will directly stimulate the proliferation of these T cells; they are no longer responsive to antigen. In addition, the TCGF assay is quantitative and sensitive.

IL2 also stimulates the AFC response of nude spleen cells to heterologous erythrocytes. It is clear, however, that IL2 is distinguishable from the late–acting TRF originally described by Schimpl and Wecker (1972). The term TRF refers to the late–acting factor, and TRF is different from IL 1 and IL2 although all 3 have helper activity for AFC responses (Swain *et al*. 1981).

By all available criteria, the activities associated with IL 1, and those associated with IL2, are mediated by a single entity each.

Recently, Mizel and Mizel (1981) reported the purification of IL 1 to apparent homogeneity. It was active at a concentration of betwen 10^{-10} and 10^{-11} M, as measured by the mitogenic response of thymocytes. No results concerning helper activity for AFC responses were reported. IL 1 was shown to have a molecular weight of 14,000 and to exhibit a microheterogeneity of 3 charge species as determined by tris–glycinate PAGE.

C. THE ROLE OF ANTIGEN-NONSPECIFIC HELPER AND PROLIFERATIVE FACTORS IN IMMUNE REGULATION

1. Interleukin 1

A variety of activities have been associated with IL 1. In addition to stimulating thymocyte proliferation, IL 1 replaces macrophages in the generation of CTLs (Farrar, W.L.

et al. 1980), and in the proliferative response of antigen-stimulated helper T cells (Mizel and Ben-Zui 1980). These 3 activities can be explained by the observation that IL 1 will stimulate the production of IL2 in macrophage-depleted cultures (Mills *et al*. 1976; Smith *et al*. 1979a, 1980b; Farrar, J.J. *et al*. 1980b; Shaw *et al*. 1980). Smith *et al*. (1979a, 1980b) demonstrated that IL 1 could restore the mitogen-induced production of IL2 in spleen cell cultures depleted of adherent cells. By using the TCGF assay, they were able to distinguish IL 1 from IL2. It was subsequently demonstrated that a murine thymic lymphoma produced IL2 in response to IL 1, thereby showing that the effect of IL 1 was mediated at the level of the T cell productivity of IL2 (Smith *et al*. 1980a).

To explain the observation that IL 1 stimulates IL2 production, Gillis and Mizel (1981) proposed that the role of IL 1 was to stimulate the maturation of a subset of T cells to the point where they are capable of IL2 production. This proposal was based on the study of a T cell lymphoma which was unresponsive to PHA stimulation in the absence of added IL 1. Gillis and Mizel demonstrated that a brief exposure of the lymphoma cells to IL 1 allowed them to produce IL2 when they were subsequently exposed to PHA. In addition, it could be shown that IL 1 was adsorbed by these lymphoma cells suggesting the presence of IL 1 receptors on them. Thus, a major role for IL 1 in the immune system may be to stimulate the maturation of IL2 producer cells.

Several other activities have also been associated with IL 1. IL 1 serves as a helper factor for AFC responses to heterologous erythrocytes (Section B3). Hoffmann *et al*. (1977) showed that IL 1 containing supernatants stimulated the differentiation of B cells (measured by the increase of surface Ia antigens). Thus, IL 1 may stimulate the maturation of both B cell and T cells. With regard to B cells, it has been proposed that IL 1 stimulates them to become responsive to TRF (Hoffmann 1980).

Another maturational function of IL 1 is suggested by the observation that IL 1 prevents the suppression of bone marrow colony formation by glucocorticoids (R.I. Mishell, personal communication). This activity is not observed with IL2.

IL 1 has also been shown to increase the membrane lipid viscosity of T cells. This increase in viscosity was correlated with an increase in antigen binding by the T cells (Puri *et al*. 1980). Lonai *et al*. (1981b) extended these observations by showing that the binding of antigen by a T cell hybridoma was dependent on IL 1. This hybridoma has been shown to

produce an antigen-specific helper factor for AFC responses (Lonai *et al.* 1981a,b). IL1 thus serves as a second signal for both IL2 production and antigen-specific helper factor production.

Finally, it appears that human IL1 and human leukocytic pyrogen are identical (Rosenwasser and Dinarello 1981). Both are adsorbed by antibodies directed against human leukocytic pyrogen, and both activities are detectable at a concentration of 10^{-12} M (which for mouse IL1 is homogeneous [Mizel and Mizel 1981]).

2. Interleukin 2

Interleukin 2 has been implicated in the stimulation of a number of immune responses both *in vitro* and *in vivo*. As previously mentioned (Section B4) IL2 appears to function as a second signal in T cell activation for both thymocyte proliferation and the generation of CTLS (Mills *et al.* 1976; Shaw *et al.* 1978a), the first signal being either antigen or mitogen.

IL2 stimulates the generation of CTLs in a variety of experimental situations, as detailed in Table 2. Of particular interest is the ability of IL2 to restore the immunogenicity of stimulator cells. Only intact, metabolically active cells stimulate CTL generation, and various treatments render these cells non-immunogenic while retaining recognizable antigen (Wagner 1973; Table 2). The observation that IL2 restores the immunogenicity of these stimulator cells suggests that they are non-immunogenic because they are unable to induce IL2 production.

In addition to augmenting CTL responses, IL2 is continually required for the maintenance of the CTLs generated in the standard CTL assay (Paetkau *et al.* 1980a). The requirement for IL2 in maintaining CTLs was first shown by Gillis *et al.* (1978a,b) with their demonstration that T cells could be grown continually in culture in the presence of IL2. IL2 also stimulates the growth and cytotoxic reactivity of natural killer cells (Kuribayashi *et al.* 1981; Henney *et al.* 1981). Recently, Farrar and co-workers (Farrar, W.L. *et al.* 1981) demonstrated that the induction of CTL responses was regulated by interferon, the production of which was mediated by IL2.

The proposal has been made by several investigators that IL2 represents the physiologically relevant 2nd signal in T cell activation. Several observations support this conclusion. The first is that IL2 is produced by Lyt 1^+ helper T cells (Wagner and

TABLE 2

GENERATION OF CTLs WITH IL2

Conditions where IL2 stimulates CTL generation	References
1. From thymocytes, in particular Lyt $1^+2^+3^+$ thymocytes	Shaw et al. 1978a Wagner et al. 1979, 1980a
2. From spleen cells, in the absence of helper T cells	Plate 1976, 1977 Shaw et al. 1980
3. In situations where the immunogenicity of the stimulators is compromised	
a) after UV irradiation, gluteraldehyde fixation, or sonication	Shaw et al. 1978a Okada et al. 1979 Wagner et al. 1980c Paetkau et al. 1980a,b
b) using membrane fragments	Rulon and Talmage 1979
c) using non-stimulating tumor cells	Lafferty et al. 1978
d) using syngeneic tumor cell lines	Mills and Paetkau 1980
e) using H-2 antigens in liposomes	Weinberger et al. 1981
4. From spleen cells obtained from nude mice	Gillis et al. 1979c Wagner et al. 1980b Gillis and Watson 1981

Rollinghoff 1978; Shaw *et al*. 1980), which are known to be involved in CTL generation (Cantor and Boyse 1975, Pilarski *et al*. 1980). These helper T cells were further shown to be Lyt 1^+ 2^-, 5^+, 6^-, 7^+, (Shaw *et al*. 1980; Pilarski *et al*. 1980). The second observation involves situations in which the immunogenicity of stimulator cells in CTL generation is lost. The inability of these cells to stimulate a CTL response is correllated with their inability to stimulate IL2 production (Okada *et al*. 1979, Paetkau *et al*. 1980a). The addition of IL2 restores their immunogenicity (for references see Table II).

The antigen-nonspecific and H-2 unrestricted nature of IL2 activity is not a strong argument against IL2 being a physiologically relevent signal. IL2 will stimulate the generation of CTLs in an antigen-nonspecific manner, yet antigen-specific helper T cells are required for CTL generation (Pilarski 1977, 1979). However, Farr *et al*. (1977) demonstrated that the production of IL1 in response to antigen-stimulated T cells required H-2 compatability between the T cells and the macrophages. As IL1 is required for IL2 production, IL2 will only be generated in an H-2 restricted manner. The generation of IL2 has also been shown to be antigen-specific. Thus IL2 is produced by KLH-primed spleen cells only when they are stimulated with KLH and not an irrelevant antigen (DiSabato *et al*. 1978). Antigen-specific production of IL2 has also been shown by Okada *et al*. (1979) and Wagner *et al*. (1980c). Thus it can be seen that the inductive stage of IL2 production is antigen-specific and H-2 restricted, although its effects are not.

The production of IL2 is also tightly regulated in another manner. Gillis *et al*. (1979a,b) demonstrated that the suppression of T cell proliferation by glucocorticosteroids was mediated at the level of IL2 production. Glucocorticosteroids were shown to inhibit IL2 production, and the effect of these steroids on T cell proliferation was abrogated by adding IL2. It is known that glucocorticosteroids injected into mice prolong the survival of skin grafts. This may, therefore, represent an in vivo mechanism for controlling the level of IL2 production.

Another mechanism for controlling IL2 activity in vivo is suggested by the observation that the serum of normal mice, but not nude mice, contains an inhibitor of IL2 activity (Hardt *et al*. 1981). This inhibitor was shown to be a 50,000 molecular weight compound produced by Lyt 2^+,3^+ T cells.

Lastly, a physiologically relevant role for IL2 is suggested by the observation that only T cells stimulated with antigen or mitogen are responsive to IL2. Thus, only activated T cells adsorb IL2 activity from the culture media. This was first shown for the continuous CTL line of Gillis *et al*. (1978b). Smith *et al*. (1979b) extended these observations and showed that spleen cells stimulated with Con A, PHA, or alloantigen, and thymocytes stimulated with Con A, adsorbed out IL2 activity. Unstimulated spleen and thymus cells, as well as LPS- stimulated spleen cells, did not adsorb out IL2 activity. These results were confirmed by several other investigators (Bonnard *et al*. 1979; Coutinho *et al*. 1979; Paetkau *et al*. 1980a).

These observations argue for a physiologically relevant role for IL2 in the activation of T cells and therefore in immune regulation.

3. The AFC Helper Activities of IL1, IL2 and TRF

The 3 soluble effectors described so far replace T cells in the AFC response of nude spleen cells to heterologous erythrocytes. The distinction between the ability of IL1 and IL2 to help an AFC response was clearly demonstrated by the experiments of Hoffmann and Watson (1979) and Hoffmann (1980). In these experiments it was shown that IL1 and IL2 exerted a marked synergy in their stimulation of the AFC response. This synergy was most evident when IL1 was added early and IL2 late. Evidence was presented that IL1 increased the number of B cells responsive to the helper activity of IL2 (Hoffmann 1980).

As mentioned previously, there is also a distinction between the helper activity of IL2 and the late-acting TRF originally described by Schimpl and Wecker (1972). The experiments of Harwell *et al*. (1980), using IL2 produced from a T cell hybridoma in response to Con A stimulation, demonstrated that IL2 did not have TRF activity when the TRF assay was rigorously depleted of both T cells and macrophages. Supernatants derived from Con A-stimulated spleen cells did however have TRF activity in this assay, suggesting the presence of a TRF which was not produced by the hybridoma and was therefore different from IL2. Harwell *et al*. (1980) proposed that the helper activity of IL2 in the standard assay system used to measure TRF activity was mediated through the stimulation of residual T cells. By eliminating these T cells, IL2 was no longer active in the AFC response. Further evidence for the distinction between the helper activity of IL2 and that

of TRF was the demonstration by Swain *et al.* (1981) of synergy between TRF and IL2. In these experiments the TRF was produced from a T cell line which did not produce either IL2 or IL1. Using an assay system thouroughly depleted of T cells and their precursors Swain *et al.* (1981) demonstrated that TRF produced from the T cell line exerted a marked synergy with IL2 in stimulating the AFC response of nude spleen cells to heterologous erythrocytes. The suggestion was made that TRF stimulated activated B cells into immunoglobulin secretion, as initially proposed by Wecker *et al.* (1975).

Another proposed mechanism for the helper activity of IL2 derives from the work of Parker and Prakash (1981). Using B cells stimulated by antibodies directed against various immunoglobulin classes, these investigators demonstrated that IL2 (in this case from the T cell hybridoma) stimulated the proliferation of activated B cells but did not stimulate their final differentiation into antibody secretion. The supernatants from Con A-stimulated spleen cells did both. The B cell growth activity of IL2 may, however, not be due to IL2 at all. Anderson and Melchers (1981) have demonstrated a 30,000 molecular weight B cell growth factor which is produced from a cloned T helper cell line. This B cell growth factor has no TCGF activity and is apparently present in Con A-stimulated spleen cell supernatants (although no data was presented). It is, therefore, conceivable that the B cell growth activity of IL2 is due to the presence of a B cell growth factor in IL2-containing supernatants and not due to IL2 (Farrar, Immunological Reviews [in press]).

Recently, a T cell hybridoma which produces TRF activity was described (Takatsu *et al.* 1980). The TRF was shown to be adsorbed by a human B blastoid cell line which was subsequently stimulated into immunoglobulin production (Muraguchi *et al.* 1981). This B cell line was not able to adsorb IL2 activity. This supports the idea that TRF directly stimulates B cells into immunoglobulin secretion and that other lymphokines which act as helper factors for B cell responses work through other mechanisms.

The proposed mechanisms by which all 3 factors exert their helper activities in the AFC response are summarized in Table 3.

TABLE 3

THE MECHANISMS OF TRF-, IL1-, AND IL2- MEDIATED HELPER ACTIVITY FOR AFC RESPONSES

Factor	Proposed Mechanism(s) of Helper Effect	References
TRF	works late, directly on B cells to stimulate immunoglobulin production.	Hunig et al. 1974 Dutton et al. 1975 Hoffmann and Watson 1979 Muraguchi et al. 1981
IL1	(a) works early, by stimulating the proliferation of residual T cells which then supply 'help' for the B cell response.	Koopman et al. 1977, 1978 Farrar, J.J., et al. 1977, 1978.
	(b) works early, through the differentiation of B cells to a level where they are responsive to T cell help.	Hoffmann and Watson 1979 Hoffmann 1980 Wood 1979a,b
IL2	(a) works early, by stimulating the proliferation of residual T cells which then supply 'help' for the B cell response.	Watson et al. 1979b Harwell et al. 1980
	(b) works early, as a B cell growth factor.	Parker and Prakash 1981 Swain et al. 1981
	(c) works late, directly on B cells to stimulate immunoglobulin production; may be due entirely to contamination of even highly purified IL2 with 'late-acting' TRF.	Hoffmann and Watson 1979 Hoffmann 1980 Harwell et al. 1980 Swain et al. 1980

II. MATERIALS AND METHODS

A. ANIMALS AND TUMOR CELL LINES

Various strains of inbred mice, as well as outbred (Swiss or ICR) mice, were used for IL2 production. All mice were maintained at the University of Alberta Health Sciences Animal Center. Breeding stock for the inbred strains of mice was originally obtained from the Jackson Laboratories, Bar Harbor, Maine.

Tumor cell lines P815 (H-2d mastocytoma), EL4 (H-2B lymphoma), and a variant of EL4 which produces IL2 (Farrar, J.J. *et al*. 1980a), were maintained by passage in tissue culture.

B. PASSAGE OF EL4 LYMPHOMA CELLS IN MICE IN THE ASCITES FORM

The IL2-producing EL4 lymphoma cells were routinely passaged in mice in the ascites form for use in the production of IL2. 2×10^6 cells in 0.2 ml of serum-free medium were injected into the peritoneal cavity of C57B1/6J mice. After approximately 2 to 3 weeks the mice were sacrificed by cervical dislocation and the EL4 lymphoma cells in the peritoneal cavity harvested. Typically, between $2 - 5 \times 10^8$ ascities cells were obtained from each mouse originally injected with 2×10^6 cells.

C. TISSUE CULTURE MEDIA AND INCUBATION CONDITIONS

RPMI 1640 medium was obtained from either Flow Laboratories, Rockville, Md., or from GIBCO Laboratories, Grand Island, N.Y., and supplemented with 20 mM sodium bicarbonate, 0.34 mM pyruvate and 0.02 M HEPES pH 7.3 (Sigma, St. Louis, Mo., Cat. #H-3375). Antibiotics, either 40 micrograms/ml gentamycin sulfate (Garamycin, Schering Corp. Ltd., Point Claire, Quebec) and 50 micrograms/ml Penicillin G potassium (10×10^4 I.U./litre, Ayerst Laboratories, Montreal, Quebec), or 50 micrograms/ml Penicillin G potassium and 74 milligrams/litre streptomycin sulfate (GIBCO), were added to the medium. This medium is referred to as RH. Media containing either 5×10^{-5} M or 1×10^{-4} M 2-mercaptoethanol are referred to as RHM, and media containing 10% fetal bovine serum (FBS) as RHF. The FBS was obtained from either Flow or GIBCO Laboratories and was inactivated at 56°C for 30 min. RH medium containing both 2-mercaptoethanol and 10%

FBS is referred to as RHFM. Some media were supplemented with heat-inactivated, gamma globulin-free, horse serum (HS, GIBCO). Media were sterilized by Millipore filtration.

Cell cultures were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C.

Some experiments were performed in minimal essential medium (MEM) supplemented with 36 mM sodium bicarbonate, 0.43 mM pyruvate and 0.025 M HEPES pH 7.3. Incubations with MEM were done in 10% CO_2.

D. PREPARATION OF SPLEEN CELLS AND IL2 PRODUCTION

Mice were killed by cervical dislocation and their spleens removed into tissue culture media. To obtain a suspension of spleen cells, the spleens were minced with scissors and teased through a stainless steel grid. Clumps were allowed to settle out for 5-10 min. The spleen cells were then washed twice in tissue culture medium by pelleting the cells at 750 x g for 7 min, decanting the supernatant, and resuspending the cells in fresh medium. Cell viability was determined with 0.14% eosin Y in saline.

For IL2 production, the spleen cells were cultured at a density of 12 x 10^6 cells/ml in RHM and stimulated with 1.5 micrograms/ml Con A (Calbiochem., La Jolla, Ca.) for 18 to 24 hr. 200-250 ml cultures were set up in Blake tissue culture bottles (surface area 250 cm^2). After incubation, the cells were pelleted at 750 x g and the supernatant decanted. This supernatant is referred to as crude IL2.

E. IL2 PRODUCTION FROM EL4 LYMPHOMA CELLS

The EL4 lymphoma cells used for IL2 production were harvested from the peritoneal cavity of mice (see section B), washed twice in tissue culture medium, and used immediately for IL2 production unless otherwise indicated. The cells were cultured at a density of 1 x 10^6 cells/ml in RH medium containing 4% HS and stimulated for 18-24 hr with PMA (Sigma) at a concentration of 10 ng/ml. Cell-free supernatants are referred to as crude IL2.

F. IL2 PRODUCTION DURING THE MIXED LEUKOCYTE REACTION

CBA/J spleen cells were cultured at a concentration of 1 x 10^6 cells/ml and stimulated with 1 x 10^6 gamma-irradiated (2000-2500 rads) DBA/2J spleen cells/ml. The cells were cultured in MEM supplemented with 5 x 10^{-5} M 2-mercaptoethanol and 10% FBS for 72 hr in a final volume of 250 ml. After incubation the cell-free supernatant was assayed for costimulator activity.

G. BIOLOGICAL ASSAYS FOR IL2

1. The Costimulator Assay

The proliferative response of thymocytes cultured at low cell density (<1 x 10^6 cells/ml) in response to Con A requires exogenous IL2 (Paetkau *et al.* 1976; Shaw *et al.* 1978b). CBA/J thymocytes, prepared as described for spleen cells (see Section D), were cultured at a density of 0.5 x 10^6 cells/ml in RHFM in 96 well, round bottom, Linbro trays. Con A was added at a final concentration of 3 micrograms/ml. The final volume of 0.2 ml included the sample of IL2 to be assayed. All samples were assayed in quadruplicate. The cultures were incubated for 67 to 72 hr at 37°C and labelled during the final 4-5 hr by adding either 100,000 cpm/well ^{125}I-UdR (Edmonton Radiopharmacy Center, 1850 Ci/mmole) or 0.05 mM ^{3}H-thymidine (Thd). The ^{3}H-Thd was from New England Nuclear (20 Ci/mmole) and was adjusted to a specific activity of 350-500 cpm/pmole with unlabelled carrier. Cultures were harvested onto glass fibre filters with a Titertek multiple sample harvester using either isotonic saline or distilled H_2O. For ^{3}H-Thd labelled cultures, the filter papers were counted using a liquid scintillation system. ^{125}I-UdR samples were counted directly in an LKB Rackgamma gamma counter.

2. Generation and Assay of CTLs

Spleen cells from CBA/J mice were cultured for 5 days in RHFM with gamma-irradiated (2,000-2,500 rads) DBA/2J spleen cells as stimulators. All cultures were performed in quadruplicate in round bottom microtitre plates in a volume of 0.22 ml. The concentration of stimulator and responder cells is given in the Figure legends.

For the assay, 3 serial 1:2 dilutions were made in round bottom microtitre plates and the target cells were added at 1 x 10^4 cells/well. The target cells used were P815 labelled with ^{51}Cr (New England Nuclear) for 2 hr at 37°C. After 5 hr at 37°C half the

supernatant was removed and the fraction of ^{51}Cr specifically released (F) was determined.

$$F = \frac{\text{(Test culture cpm - background)}}{\text{(Total lysis cpm - background)}}$$

The total lysis was determined by adding 1:2000 (V/V) "Zap-Isoton" (Coulter Electronics, Hialeah, Fl.). The data are expressed as % specific release at a particular ratio of effector to target cells, as indicated in the Figure legends.

In some experiments, thymocytes were used as responders and EL4 lymphoma cells as both stimulators and targets. In all cases, the targets were the same H-2 type as the stimulator cells.

3. TCGF Assay

The TCGF assay, performed according to Gillis *et al*. (1978b), measures the requirement of certain types of T cells grown continually in culture for IL2. The cells used for the TCGF assay were MTL 2.8.1 (Bleackley *et al*. 1982). These cells were obtained by the repeated restimulation in vitro of in vivo primed CBA/J spleen cells with gamma-irradiated (BALB/cCr x CBA/CaJ) F_1 spleen cells. MTL 2.8.1 was cloned by limiting dilution. The characterization of MTL 2.8.1 cells has recently been described (Bleackley *et al*. 1982).

Before use in the TCGF assay, cells were routinely cultured at 40,000 cells/ml for 3 days in the presence of 30 TCGF units/ml IL2. During the 3 day culture period these cells increase in number 10-15 fold and deplete the medium of IL2. They are then ideal for use in the TCGF assay as they die within 24 hr if placed in medium lacking IL2.

For the assay, 1 x 10^4 cells/well were cultured in flat bottom microtitre plates in 0.2 ml RHFM. The cells were cultured for 24 hr and labelled for the last 4-6 hr with ^{125}I-UdR as described for the costimulator assay. The labelling mixture contained 0.1 mM deoxyinosine and 5 micrograms/ml fluorodeoxyuridine. Only very low concentrations (ca. 10^{-10}M) of ^{125}I-UdR were present in the assay and it was observed that hydrolysis of the ^{125}I-UdR occurred in cultures of MTL 2.8.1 cells. The combination of deoxyinosine and fluorodeoxyuridine blocks the hydrolysis of ^{125}I-UdR and inhibits endogenous synthesis of thymidine triphosphate, both resulting in enhanced uptake of label.

To harvest the cells, the supernatant was aspirated and the cells resuspended in 20 mM EDTA in phosphate-buffered saline. The cells were incubated for 5 min at room temperature and then harvested onto glass fibre filters as described for the costimulator assay. The incubation of the cells in EDTA in phosphate-buffered saline was necessary because the MTL 2.8.1 cells adhered to the plastic microtitre plates and were not harvested unless first forced into suspension.

Some of the TCGF assays were performed using uncloned cells at various stages of in vitro culture. In these assays labelling was with ^{125}I-UdR in the absence of deoxyinosine and fluorodeoxyuridine. Comparable results were obtained in all cases.

H. PURIFICATION OF IL2 PRODUCED BY NORMAL SPLEEN CELLS

1. Ammonium Sulfate Precipitation

Ammonium sulfate precipitation was performed at 80-90% saturation. Ammonium sulfate was added slowly to the culture supernatants with gentle stirring. After 15 hr at 4°C, the precipitate was collected by centrifugation for 15 min at 10,000 x g or for 1 hr at 4,000 x g. The precipitate was redissolved in a small volume of distilled H_2O.

2. Sephadex G-100 Chromatography

A 2.5 x 90 cm column (500 ml) of Sephadex G-100 (Pharmacia) was equilibrated in buffer A - 0.05 M NaCl, 0.01 M HEPES pH 7.3. The void and total volumes of the column were determined using Blue Dextran 2000 and ^{3}H-Thd respectively. Samples applied to the column ranged in volume from 10-50 ml. Chromatographic runs were done at 4°C.

3. Sephacryl S-200 Chromatography

A 0.9 x 100 cm column (130 ml) of Sephacryl S-200 (Pharmacia) was equilibrated in 0.25 M NaCl, 0.01 M HEPES pH 7.3. The void and total volumes of the column were obtained using ^{3}H-DNA and ^{14}C-Thd respectively. Samples of 1-4 ml were applied to the column. Chromatographic runs were done at 4°C.

4. DEAE-Sephacel Chromatography

Preswollen DEAE-Sephacel (Pharmacia) was equilibrated in buffer A (section H2) containing 0.2 mM EDTA and transferred to a 0.9 x 15 cm column (8 ml). Fraction 3 IL2 (purified by Sephadex G-100 chromatography) was applied to the column and the column washed extensively with starting buffer. Activity was eluted with a linear salt gradient of

0.05 M to 0.35 M NaCl. Fractions of peak activity were pooled and are referred to as fraction 4 IL2. Chromatographic runs were done at 4°C.

I. PURIFICATION OF IL2 PRODUCED BY EL4 LYMPHOMA CELLS

1. Ammonium Sulfate Precipitation

Ammonium sulfate precipitation was performed as described in Section H1 except that 1–2 hr at 4°C was sufficient to precipitate the protein. The precipitate was redissolved in a small volume of buffer A.

2. Sephadex G-100 Chromatography

Sephadex G–100 chromatography was performed as described in Section H2.

3. Phenyl-Sepharose Chromatography

Preswollen Phenyl–Sepharose (Pharmacia) was equilibrated in buffer A containing 2.5 M NaCl and transferred to a 2.5 x 20 cm column. The packed bed volume was 100 ml. Fraction 3 IL2 (purified by G–100 chromatography) was made 2.5 M in NaCl by slowly adding solid NaCl with stirring at 4°C. After application of the sample, the column was washed extensively with starting buffer. Activity was eluted from the column with a linear gradient of buffer A containing 2.5 M NaCl to buffer A containing 50% ethylene glycol. In some experiments activity was eluted from the column by washing with buffer A containing 10% ethylene glycol. Chromatographic runs were done at 4°C.

4. DEAE-Sephacel Chromatography

DEAE–Sephacel chromatography was performed as described in Section H4 except that a much larger column (total bed volume of 80 ml) was used. IL2 was purified by Sephadex G–100 and Phenyl–Sepharose chromatography and dialyzed against buffer A before being applied to the column.

5. Chromatofocusing

Chromatofocusing is a technique recently developed by Pharmacia which separates proteins on the basis of their isoelectric points. This technique uses an anion exchange resin and ampholytes. Unlike isoelectric focusing, no electric current is required. The chromatofocusing technique is described in a pamphlet supplied by Pharmacia.

The anion exchange resin PBE 94 was obtained preswollen and was equilibrated in 0.025 M piperazine–HCl pH 5.5, and transferred to a 1 x 40 cm column. The packed bed

volume was 25 ml. Before use the resin was equilibrated by washing with 10 column volumes of 0.025 M piperazine–HCl pH 5.5. The IL2 used for the chromatofocusing was applied to the column in a volume less than 10 ml in low salt buffer, typically 0.01 M NaCl, 0.01 M HEPES pH 7.3.

To fractionate the IL2 according to its isoelectric point(s), the chromatofocusing column was eluted with ampholytes. The ampholytes used were Polybuffer 74 which had been titrated to a pH between 2.8 to 3.5 with 0.5 M HCl. After titration the ampholytes were diluted 10 fold. Approximately 10 column volumes of diluted ampholytes were required for the chromatofocusing run. After the elution with ampholytes, any remaining material bound to the resin was washed off with 1 M NaCl.

Typically, the fractionation was performed at a flow rate of 10–15 ml/hr. 4–5 ml fractions were collected and the pH of each fraction was measured. After its pH was measured, each fraction received buffer with a pH of 7.3–7.4 to raise the pH.

All buffers used in the chromatofocusing run were degased before use. The experiments were performed at room temperature and required overnight elution.

After use, the resin was re–equilibrated by washing extensively in 0.025 M piperazine–HCl pH 5.5. It was then ready to be re–used.

J. DENATURATION OF IL2 WITH SDS AND RECOVERY OF ACTIVITY

IL2 was treated with 1.0% SDS (Bio–Rad, Richmond, Ca., electrophoresis grade) at the temperatures and for the times indicated. Typical conditions were 70°C for 10 min. After denaturation the samples were diluted 10 fold to give a concentration of 0.1% SDS, and cooled to 0°C. This procedure precipitated free SDS. The samples were then centrifuged at 0°C and the supernatant was carefully pipetted off. Any remaining SDS was reduced in concentration by dilution with RHF or RHFM immediately before assay.

K. SDS-PAGE

Partially purified IL2 was fractioned by SDS–PAGE using either the sodium phosphate buffer system or the Laemmli discontinuous buffer system (Laemmli 1970).

Using the sodium phosphate buffer system, IL2 was fractionated in a slab gel with an acrylamide concentration of 10%. The stock acrylamide solution was 30% by weight

acrylamide and 0.8% by weight N,N'-bis-methylene acrylamide. The final concentrations in the gel were 0.05 M sodium phosphate pH 7.2, 0.1% SDS.

Laemmli gels consisted of a 3% stacking gel in 0.125 M Tris-HCl pH 6.8, 0.1% SDS and a 10% separating gel in 0.375 M Tris-HCl pH 8.8, 0.1% SDS. The electrode buffer contained 0.025 M Tris, 0.192 M glycine pH 8.3, 0.1% SDS.

In all cases, the acrylamide was polymerized with 0.025% by volume TEMED and 0.1% ammonium persulfate. Electrophoresis was performed at room temperature for 6-7 hr at approximately 300 volts. Samples were denatured as indicated in the Figure legends.

The behaviour of IL2 was determined by assaying the material which was eluted from the gels (as described in the Figure legends). MW markers used were bovine serum albumin (67,000), ovalbumin (43,000), chymotrypsinogen A (25,000) and cytochrome C (13,000).

L. GEL FILTRATION CHROMATOGRAPHY OF IL2 AFTER SDS DENATURATION

A 0.9 x 100 cm column (60 ml) of Sephacryl S-300 (Pharmacia) was equilibrated in buffer A containing 0.2 mM EDTA, 0.1% SDS and 1 mM dithiothreitol (DTT). The column was calibrated with the molecular weight markers as described in Section K, and including phosphorylase a (94,000). All proteins were incubated at 70°C for 10 min in the presence of 1% SDS and 10 mM DTT before being applied to the column. The void and total volumes of the column were determined using Blue Dextran 2000 and ^{3}H-Thd, respectively.

IL2 was incubated at 70°C for 10 min in the presence of 1.0% SDS and 10 mM DTT. Either 1,000 costimulator units of IL2 from Con A-stimulated spleen cells or 14,000 TCGF units from PMA-stimulated EL4 lymphoma cells were used. After being cooled to room temperature, the samples were applied to the column and eluted with buffer A containing 0.2 mM EDTA, 0.1% SDS and 1 mM DTT. In experiments performed without DTT treatment, DTT was also omitted from the elution buffer. All chromatographic runs were performed at room temperature. Free SDS was removed as described in Section J.

M. GLYCEROL GRADIENT CENTRIFUGATION OF IL2 AFTER SDS DENATURATION

5-20% glycerol gradients were prepared in 0.02 M HEPES pH 7.3, 1 mM EDTA, 0.1% SDS, and 2 mM DTT. Gradients were run in polyallomer tubes in a Beckman SW-65 (swinging bucket) rotor at 60,000 rpm for 16-17 hr at 15°C. Samples were denatured and reduced before being applied to the gradient. BSA was present in all gradients as a marker. The gradient fractions were assayed for costimulator activity after a 5-fold dilution into RHF medium.

N. GAMMA IRRADIATION OF CELLS

Cells were irradiated in a ^{137}Cs source (Gamma cell 40, Atomic Energy of Canada Ltd.) at room temperature. The dose of irradiation given to cells was either 2000 or 2500 rads. Cells were irradiated in plastic tissue culture tubes in RHFM.

O. UV IRRADIATION OF CELLS

Spleen cells were UV irradiated in 32 mm petri dishes for 2 min at a distance of 10 cm from a UV germicidal lamp (General Electric, 15 watt bulb). The cells were at a density of 10×10^6 cells/ml in a volume of 1 ml.

P. PROTEIN DETERMINATION

Protein determinations were carried out by the sensitive Coomassie Blue dye method (Bradford 1976). Samples ranged in volume from 0.02 ml to 0.1 ml and reagent from 0.5 ml to 1.5 ml. The absorbance at 595 nm was determined.

III. STUDIES ON IL2 PRODUCED FROM NORMAL MURINE SPLEEN CELLS

A. INTRODUCTION

When murine spleen cells are stimulated in vitro with the mitogenic lectin Con A they secrete a number of immunostimulatory factors, one of which allows thymocytes cultured at low cell density to proliferate in response to Con A. This factor does not, in itself, stimulate thymocyte mitogenesis but rather serves as a costimulator with Con A. It was therefore referred to as costimulator (see Chapter I, Section B4b).

In addition to costimulator, other factors with various immunostimulatory activities were present in the supernatants of Con A-stimulated spleen cells. When these factors were characterized biochemically, the similarity between several of them became apparent. It was subsequently realized that several of the activities in supernatants of Con A-activated spleen cells were mediated by a single entity. This entity was named Interleukin 2 (IL2) based on its ability to serve as a signal between leukocytes (see Chapter I, Section B4d). The designation IL2 includes the factor initially referred to as costimulator. The ability of IL2, in the presence of Con A, to stimulate the proliferation of thymocytes cultured at low cell density will be referred to as costimulator activity.

Using the costimulator assay, experiments were initiated to purify IL2 from the supernatants of Con A-stimulated spleen cells. The initial fractionation of IL2 in this lab was performed by Dr. Jennifer Shaw and involved concentrating the supernatants by lyophilization, Sephadex G-25 chromatography and then Sephadex G-100 chromatography. A procedure for purifying large amounts of supernatant material is reported in this chapter. Also reported are studies on the properties of IL2 denatured with SDS, and experiments to study the role of IL2 in the MLR.

B. RESULTS

1. The Costimulator Assay for IL2

IL2 can be measured by its ability to stimulate low density cultures of thymocytes to proliferate in response to Con A. This activity is referred to as costimulator. The assay is described in detail in Materials and Methods. Briefly, thymocytes are cultured at a density of 0.5×10^6 cells/ml in RHFM and stimulated for 68 to 72 hr with 3.0 micrograms/ml Con

A and the sample of IL2 to be assayed. During the last 4 to 6 hr of culture the thymocytes are labelled with either ^{3}H-Thd or ^{125}I-UdR, the incorporation of which is a measure of the extent of cellular proliferation. This assay has previously been shown to provide a quantitative measurement of the amount of IL2 present in a particular sample (Paetkau *et al.* 1976, Shaw *et al.* 1978b).

It can be seen in Figure 1 that over a certain range of concentration there is a direct relationship between the amount of partially purified IL2 added to the cultures and the amount of thymocyte proliferation observed. Identical results are obtained when either ^{3}H-Thd or ^{125}I-UdR is used to measure the extent of proliferation. Samples labelled with ^{125}I-UdR can be counted without the addition of scintillation fluid thus facillitating their handling.

The dilution curve depicted in Figure 1 can be used to determine the number of units of IL2 present in the sample assayed. The concentration of IL2 which will induce 1/3 maximum response in a particular assay has been defined as 1 unit/ml. In Figure 1, 1/3 of the maximum response occurs at a dilution of approximately 700. There are, therefore, 700 units/ml IL2 in this sample.

The amount of costimulator activity present in a particular sample can be expressed in one of three ways: (1) pmoles of ^{3}H-Thd incorporated /hr/ 10^{4} cells; (2) % maximal stimulation at a particular dilution, with maximal stimulation being determined in the presence of optimal IL2, and (3) units of costimulator activity. All 3 designations facilitate a direct comparison within and between experiments.

Two other systems used to assay IL2 activity are the continuous proliferation of T cells (TCGF assay) and the generation of CTLs (see Materials and Methods). These assays differ in their sensitivity to IL2. The TCGF assay is the most sensitive: approximately 4 fold more than the costimulator assay and approximately 8 fold more than the assay measuring the generation of CTLs. (data not shown).

2. The Production and Purification of IL2

For the large scale production of IL2, murine spleen cells were cultured at a concentration of 12 x 10^{6} cells/ml in RHM medium (no serum) containing 1.5 micrograms/ml Con A for 18-24 hr at 37°C. After incubation, the cells were pelleted by centrifugation and the supernatant decanted. This supernatant is referred to as crude IL2.

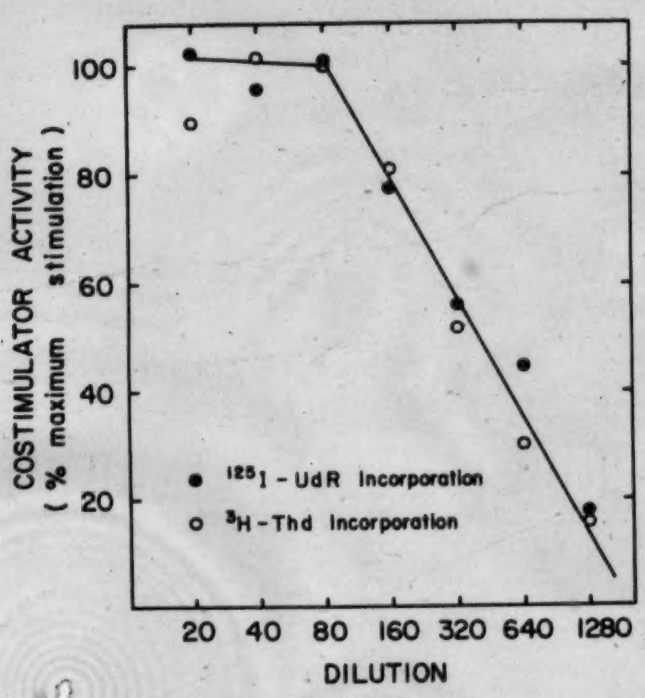

Fig. 1 - The costimulator assay for IL2: Comparison of ^{125}I-UdR and ^{3}H-Thd incorporation. CBA/J thymocytes were cultured at a density of 0.5 x 10^{6} cells/ml in RHFM and stimulated with 3.0 micrograms/ml Con A and various dilutions of IL2, as described in Materials and Methods. The cultures were incubated for 67 to 72 hours and labelled during the last 4-5 hours with either 100,000 cpm ^{125}I-UdR or 460,000 cpm ^{3}H-Thd. Results are expressed as a percent of the maximum stimulation observed with an optimal amount of IL2. In this experiment, maximum stimulation resulted in the incorporation of 17,000 cpm of ^{125}I-UdR (17%) and 68,000 cpm of ^{3}H-Thd (15%). The IL2 used was purified by ammonium sulfate precipitation and gel filtration chromatography as described in Materials and Methods.

Crude IL2 was usually obtained in volumes ranging from 1-5 litres. These supernatants were precipitated with ammonium sulfate at 90% saturation and the protein collected by centrifugation. This procedure brings down the majority of protein and IL2 present in the crude supernatants. Precipitation with ammonium sulfate was easier to handle on a large scale than was freeze drying. Following ammonium sulfate precipitation the IL2 was dialyzed (fraction 2) and chromatographed on a Sephadex G-100 column. Costimulator activity eluted with proteins of apparent MW between 30,000 and 45,000. Active fractions were pooled to give fraction 3 material.

Fraction 3 IL2 was further purified by ion-exchange chromatography on DEAE-Sephacel. A representative experiment is shown in Figure 2. Activity eluted at a salt concentration of 0.12 - 0.14 M NaCl, with no more activity eluting at salt concentrations up to 1 M NaCl. Active fractions were pooled generating fraction 4 IL2.

Figure 2 also illustrates that the costimulator activity of IL2 and an activity which stimulates the generation of CTLs from thymocyte precursors co-purify through ion-exchange chromatography. This supports the conclusion reached earlier (Shaw *et al.* 1978b) that the same entity is responsible for both activities.

A summary of this purification protocol is given in Table 4. The most efficient purification step was the DEAE- Sephacel chromatography. Typically a 5-10 fold increase in specific activity was observed with essentially quantitative recovery of activity.

3. Studies on SDS-Denatured IL2

Quantitative recovery of IL2 activity was observed after SDS denaturation if the free SDS was precipitated at 0°C (see Table 5). Previous experiments (data not shown) suggested that free SDS killed the thymocytes used to measure IL2 activity. The precipitation of free SDS by cooling the samples to 0°C, followed by dilution into medium containing FBS thus efficiently removes the free SDS. When IL2 was denatured in the presence of 10 mM 2-mercaptoethanol or 10 mM dithiothreitol (DTT), a lower recovery of activity was observed (about 50%). However, this may reflect the carryover of the thiol into the assay at inhibitory concentrations and not a decreased amount of IL2 (results not shown).

The ability to assay IL2 after SDS denaturation allowed us to perform experiments on the properties of SDS-denatured IL2. Figure 3 shows the results obtained when

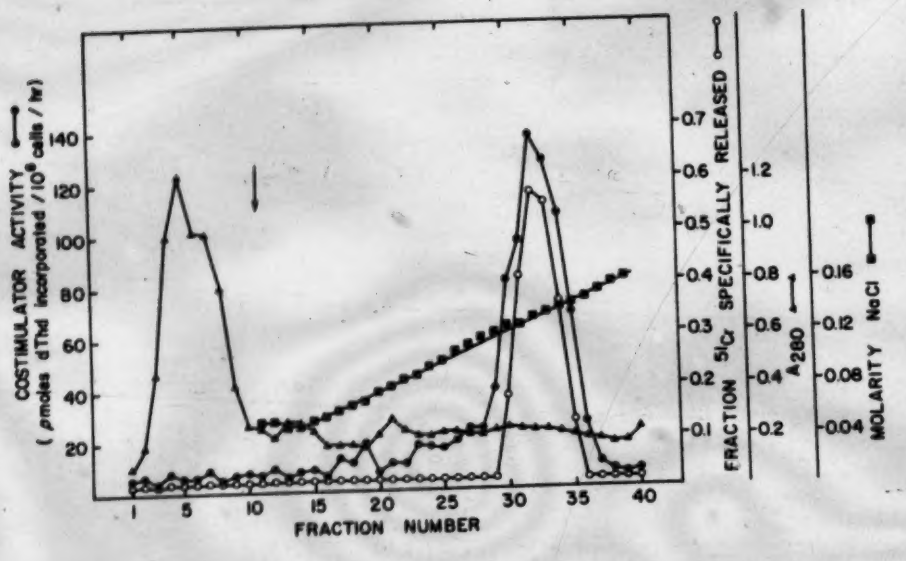

Fig. 2 – Chromatography of fraction 3 IL2 on DEAE–Sephacel. Fraction 3 IL2 was prepared from spleens of outbred (Swiss) mice and chromatographed on DEAE–Sephacel as described in Materials and Methods. The point at which the salt gradient (0.05–0.35 M) was applied is indicated with an arrow. Fractions (4.4 ml) were assayed for both costimulator activity (at a 1/50 dilution) and for activity in generating CTLs (at 1/40 dilution). The generation and assay of CTLs was performed as described in Materials and Methods using 3×10^5 CBA/J thymocytes as responders and 3×10^3 gamma–irradiated EL4 tumor cells as stimulators.

TABLE 4

PURIFICATION OF IL2[a]

STAGE	PURIFICATION STEP	VOLUME (ml)	PROTEIN (mg)	ACTIVITY[b] (units)	SP. ACT units/mg protein
Crude	-	4,000	359	64,000	180
Fract 2	Ammonium sulfate precipitation	55	104	18,500	170
Fract 3	Sephadex G-100 chromatography	160	71	19,000	270
Fract 4	DEAE-Sephacel chromatography	20	13	19,000	1,500

(a) see text for explanation.

(b) activity was determined using the costimulator assay.

TABLE 5

RECOVERY OF IL2 AFTER SDS DENATURATION[a]

Treatment	Costimulator Activity[b]	% Recovery
Control	90	100
1% SDS, 90°C for 2 min.	120	133
1% SDS, 70°C for 10 min.	80	89

(a) fraction 4 IL2 was treated with 1% SDS as indicated and the free SDS was precipitated at 0°C. After centrifugation the supernatant was assayed for costimulator activity.

(b) activity is expressed as the average number of units in duplicate samples.

SDS-denatured IL2 was fractionated by PAGE using the discontinuous buffer system of Laemmli (Laemmli 1970). IL2 had a mobility characteristic of proteins of 25,000 MW. Similar results were obtained with IL2 denatured in the presence of reducing agent, although the recovery of activity was less. Also illustrated in Figure 3 is that background responses for CTL generation were not inhibited by the samples which were assayed. The somewhat higher MW of 30,000 was obtained when SDS-denatured IL2 was fractionated by SDS-PAGE using the sodium phosphate buffer system, as illustrated in Figure 4. Activities stimulating the generation of CTLs (Figure 3) and the continuous proliferation of CTLs in long term culture (TCGF activity, Figure 4), co-purified with costimulator activity. In both experiments IL2 was denatured by treatment with 1% SDS at room temperature for 1 hr. When IL2 was denatured in 1% SDS at 70°C for 10 min, and then fractionated by SDS-PAGE (again using the Laemmli buffer system), it had a mobility characteristic of proteins of less than 20,000 MW (Figure 5). The precise MW of IL2 in this experiment could not be determined as proteins with MWs as high as 13,000 (as illustrated by cytochrome c) ran with the ion front and did not seperate out according to MW. In all 3 SDS-PAGE experiments, less than 10% of the activity applied to the gel was recovered (data not shown). This observation, along with the different results obtained in the 3 experiments, made it difficult to assign a MW to SDS-denatured IL2. A further problem in determining the MW of IL2 by SDS-PAGE is that IL2 is a glycoprotein. Glycoproteins give anomalously high MW values when characterized by SDS-PAGE (Segrest and Jackson 1972, see Discussion).

To overcome the problems associated with SDS-PAGE experiments the molecular size of SDS-denatured IL2 was determined by gel filtration chromatography. Figure 6A is the elution profile of SDS- and DTT-treated standard proteins and IL2 from a Sephacryl S-300 column. The elution buffer (0.05 M NaCl, 0.01 M HEPES pH 7.3, 0.02 mM EDTA) contained 0.1% SDS and 1 mM DTT to ensure that no renaturation or disulfide bond formation occurred during the run. A linear relationship was obtained between the partition coefficient, K(av), and the log of the MW of all 5 standard proteins (Figure 6A). The standard proteins, and IL2, were treated with 1% SDS at 70°C for 10 min in the presence of 10 mM DTT to ensure complete denaturation.

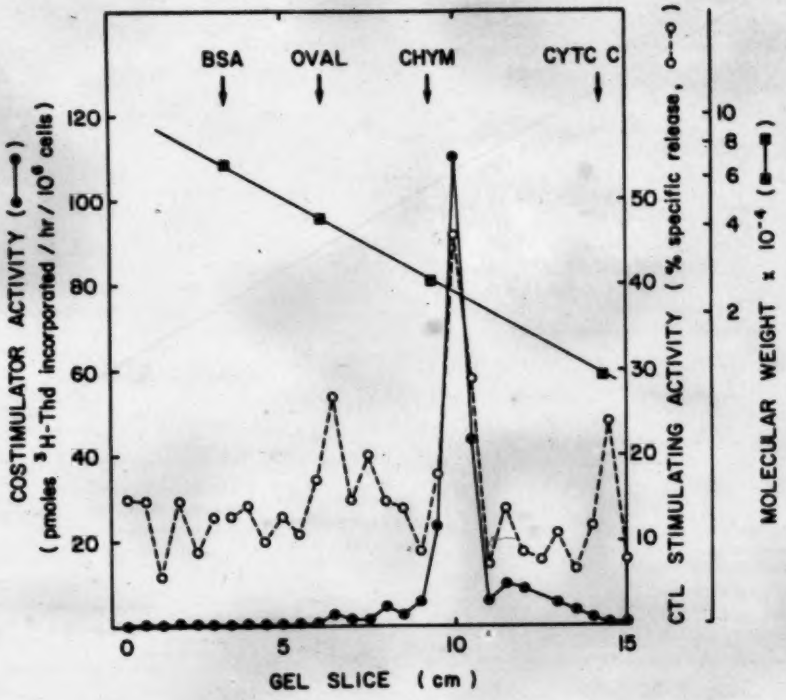
BSA
OVAL
CHYM
CYTC C
COSTIMULATOR ACTIVITY (●—●)
(pmoles ³H-Thd incorporated / hr / 10⁶ cells)
CTL STIMULATING ACTIVITY (% specific release, o--o)
MOLECULAR WEIGHT x 10⁻⁴ (■—■)
GEL SLICE (cm)

Fig. 3 - SDS-PAGE of IL2 using the Laemmli buffer system. Fraction 4 IL2 was denatured at room temperature for 1 hr in the presence of 1% SDS and fractionated by PAGE in a 10% acrylamide slab gel using the discontinuous buffer system of Laemmli (see Materials and Methods). After electrophoresis the gel was sliced into 5 mm pieces and each piece was minced in buffer A containing 20 micrograms/ml hemoglobin as carrier protein. The minced gel slices were incubated overnight at room temperature to allow diffusion of the IL2 into the buffer. After centrifugation to remove the residual acrylamide, the supernatant was cooled on ice to precipitate free SDS. The samples were assayed for costimulator activity at a final dilution of 1/10, and for CTL stimulating activity at a final dilution of 1/5. (■) MW calibration curve, markers are bovine serum albumin (BSA), ovalbumin (OVA), chymotrypsinogen A (CHYM) and cytochrome c (CYTC C). The markers were denatured in 1% SDS for 1 hr at room temperature in the presence of 10 mM 2-mercaptoethanol. CTLs were generated from 2.5 x 10^5 CBA/J spleen cells stimulated with an equal number of gamma-irradiated DBA/2J spleen cells. CTLs were assayed on 1 x 10^4 target cells/well.

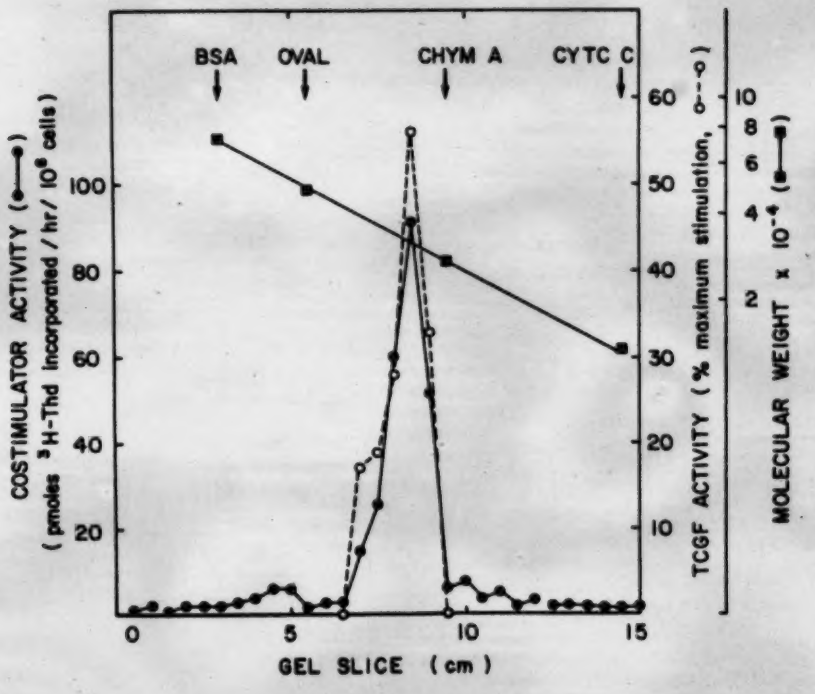
BSA
OVAL
CHYM A
CYTC C
COSTIMULATOR ACTIVITY (●—●)
(pmoles ³H-Thd incorporated / hr/ 10⁵ cells)
TCGF ACTIVITY (% maximum stimulation, o--o)
MOLECULAR WEIGHT x 10⁻⁴ (■—■)
GEL SLICE (cm)

Fig 4 - SDS-PAGE of IL2 using the sodium phosphate buffer system. Fraction 4 IL2 was denatured as described in Figure 3, and fractionated on a 10% acrylamide gel using the sodium phosphate buffer system (see Materials and Methods). After electrophoresis the gel was sliced into 5 mm pieces and each piece was placed in a sealed eppendorf tip containing a siliconized glass wool plug. The gel slice was minced in buffer (0.01 M HEPES pH 7.3, 0.5 M NaCl, 0.2 mM EDTA) containing 20 micrograms/ml hemoglobin, and incubated overnight at room temperature and then for 1 hr at 37°C. After incubation, the tips of the eppendorf tubes were cut, the tubes were centrifuged and the supernant collected. The presence of IL2 was assayed in the costimulator and TCGF assays at a final dilution of 1/10, after the removal of free SDS by precipitation. The TCGF assay was performed using a continuous cell line which had been in culture for 10 days. (■) MW calibration curve, markers as in Figure 3.

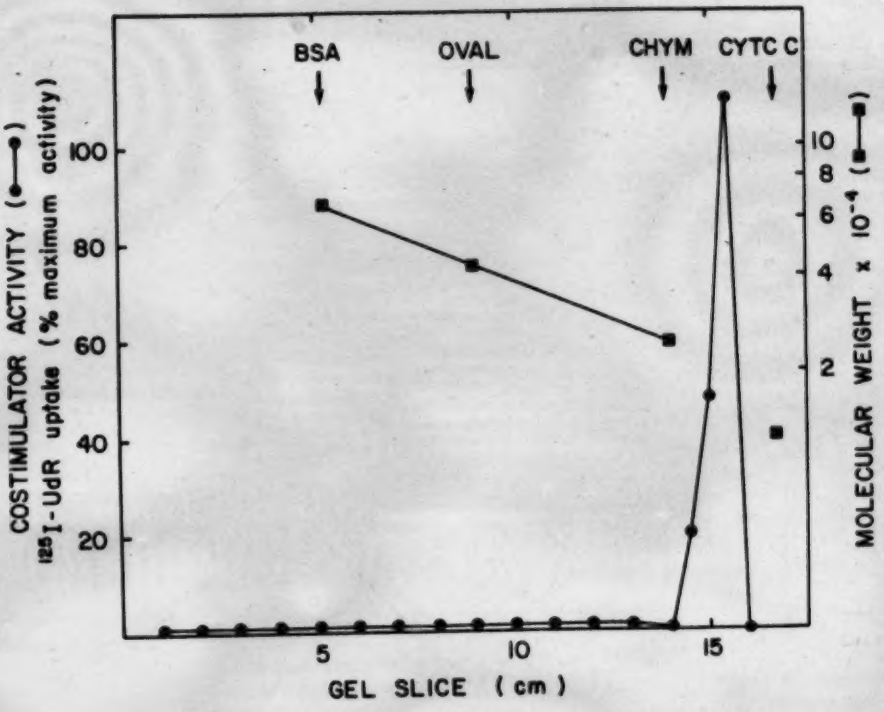

Fig. 5 - SDS-PAGE of IL2 using the Laemmli buffer system: Experiment 2. Fraction 4 IL2 was denatured by heating at 70°C for 10 min in the presence of 1% SDS and then fractionated by SDS-PAGE as described for Figure 3. IL2 was eluted and assayed for costimulator activity at a final dilution of 1/10. (■) MW calibration curve, markers as in Figure 3. The markers were denatured in 1% SDS for 10 min at 70°C in the presence of 10 mM DTT.

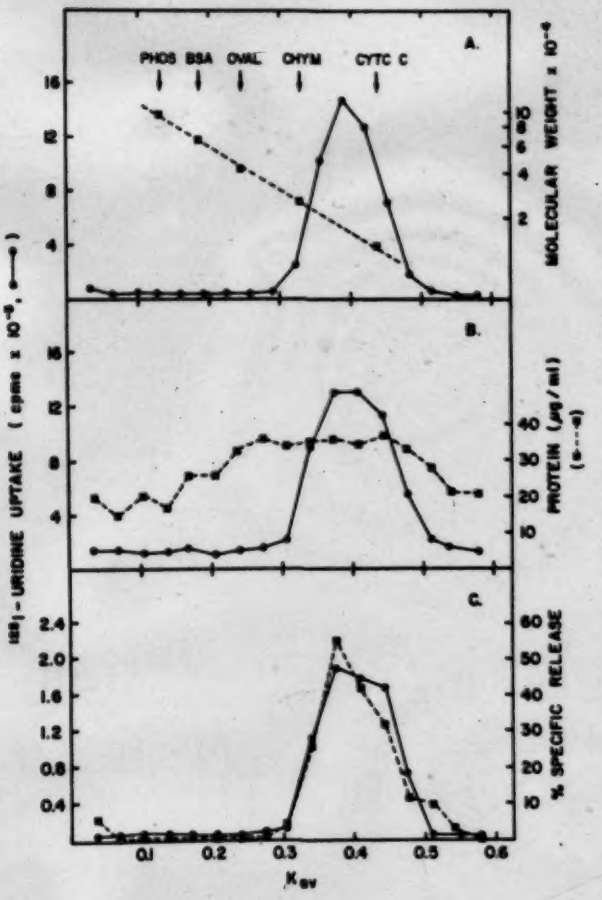

Fig 6 – Gel filtration chromatography of IL2 after SDS denaturation. SDS-denatured IL2 was chromatographed on a Sephacryl S-300 column in buffer containing 0.1% SDS with or without 1 mM DTT (see Materials and Methods). A. Fraction 4 IL2 was denatured with 1% SDS in the presence of 10 mM DTT for 10 min at 70°C. (■) MW calibration curve, markers as in Figure 3, and including phosphorylase a (PHOS); (●) costimulator activity at 1/20 dilution after removal of free SDS by precipitation at 0°C. B. IL2 was denatured in the absence of DTT; (■) protein concentration; (●) costimulator activity at 1/50 dilution after the removal of free SDS. C. IL2 was denatured in the absence of DTT; (■) generation of CTLs at 1/30 dilution after removal of free SDS. 5 x 10^5 CBA/J spleen cells were used as responders and 5 x 10^5 gamma-irradiated DBA/2J spleen cells were used as stimulators; (●) TCGF activity at 1/20 dilution after removal of free SDS. At the time of assay the cells used for the TCGF assay had been in culture for 1 month.

The costimulator activity of IL2 chromatographed with proteins of 16,000 ± 1,500 MW (average of 3 experiments). There was no indication of costimulator activity in the molecular weight range of 30-45,000. Figures 6B and 6C illustrate that DTT treatment had no effect on the elution profile of SDS-denatured IL2. Helper activity for CTL generation and TCGF activity co-purified with costimulator activity (Figures 6B and 6C). All 3 activities co-purified whether or not DTT was present. The recovery of IL2 activity after gel filtration was typically 50%.

This gel filtration column was also used to determine the MW of the low-MW form of IL1 produced from LPS-stimulated P388D$_1$ cells. The MW obtained was 18,500 (data not shown), somewhat higher than the MW of 12-16,000 reported by Mizel (1979).

The behaviour of SDS-denatured IL2 during glycerol gradient centrifugation was also determined. Figure 7 illustrates that SDS- and DTT- treated IL2 sedimented with proteins of MWs centered around 18,000 (average of 3 experiments). This value was obtained using a 5-20% glycerol gradient in buffer containing SDS and DTT. The sedimentation velocity of SDS-denatured IL2 was determined relative to that of BSA which was present in each gradient. A linear relationship was obtained between the log of the molecular weights of the 5 standard proteins and their relative sedimentation velocity (Figure 7). This 18,000 MW value agrees reasonably well with the values of 16,000 obtained from gel filtration studies. These two techniques, taken together, provide a MW value for SDS-denatured IL2 which is independent of shape (Siegel and Monty 1966, see Discussion).

4. The Production of IL2 During a MLR and its Abrogation by UV Irradiation

Costimulator activity is produced during the MLR (Shaw *et al.* 1978a). Figure 8 demonstrates that the costimulator activity derived from both Con A- and MLR-stimulated spleen cells behaves similarly during Sephacryl S-200 chromatography.

When the stimulator cells in the MLR are UV irradiated the amount of IL2 produced is dramatically decreased (Figure 9). At 96 hours after the initiation of culture there is a 10 fold difference in the amount of costimulator activity produced when the stimulators are UV irradiated. In addition, the proliferative response of spleen cells stimulated in the MLR is almost completely abrogated by UV irradiation, as demonstrated in Figure 10 (compare the open circles in Figures 10A and 10B). The addition of exogenous IL2, in this case fraction

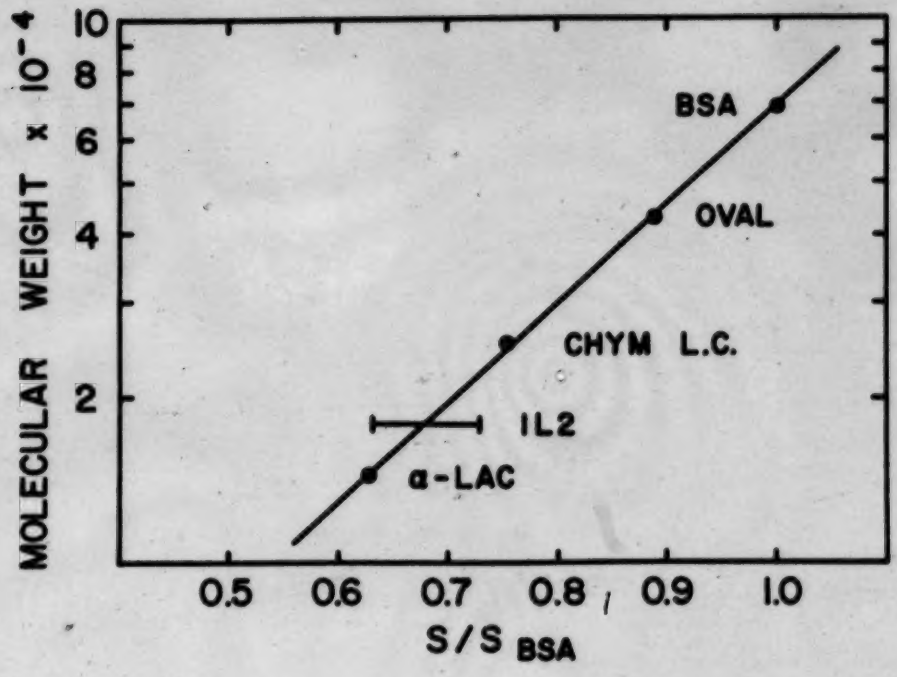

Fig. 7 - Sedimentation velocity of IL2 after SDS denaturation. Samples were treated with SDS and DTT as described in Materials and Methods and applied to a 5-20% glycerol gradient. Centrifugation was for 15-16 hr at 60,000 rpm at 15°C. The positions of marker proteins are expressed relative to that of BSA, which was present in each gradient. Markers as in Figure 3 and including immunoglobulin light chain (L.C.) and alpha-lactalbumin (alpha-LAC). The range of sedimentation values for IL2 was obtained from 3 separate centrifugation runs.

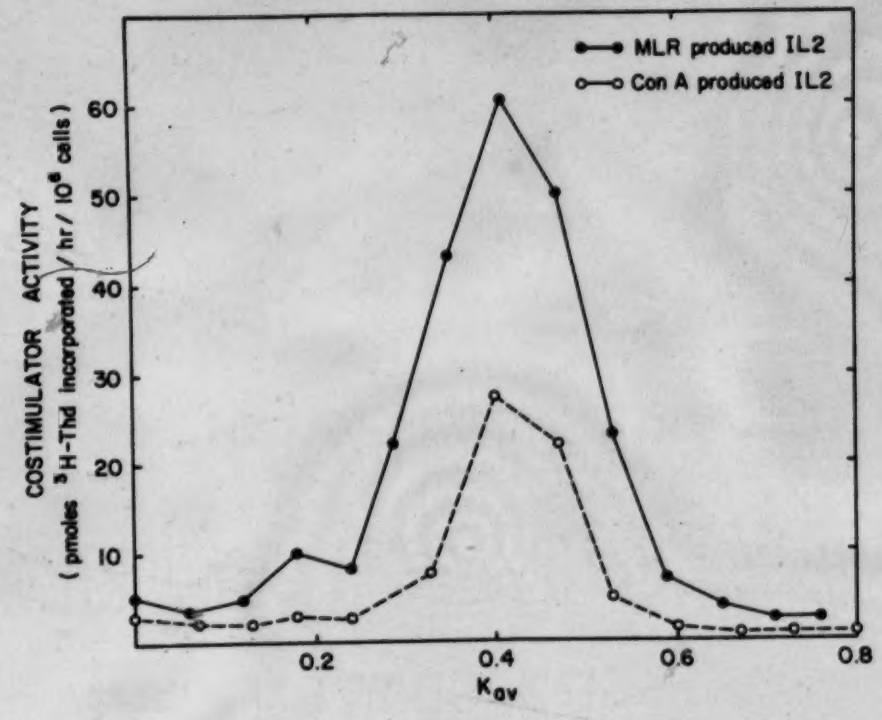

Fig. 8 - Gel filtration chromatography of IL2 produced by Con A- and MLR-stimulated spleen cells. IL2 was prepared from Con A-stimulated spleen cells and from an MLR between CBA/J and BALB/C spleen cells as described in Materials and Methods. The crude supernatants were ammonium sulfate precipitated and then chromatographed on a Sephadex G-25 column to remove salts and Con A. After lyophilization to concentrate the samples, they were chromatographed on a calibrated Sephacryl S-200 column. ^{3}H-DNA and ^{14}C-Thd were used to determine the excluded and included volumes, respectively. Fractions were assayed for costimulator activity at 1/10 dilution.

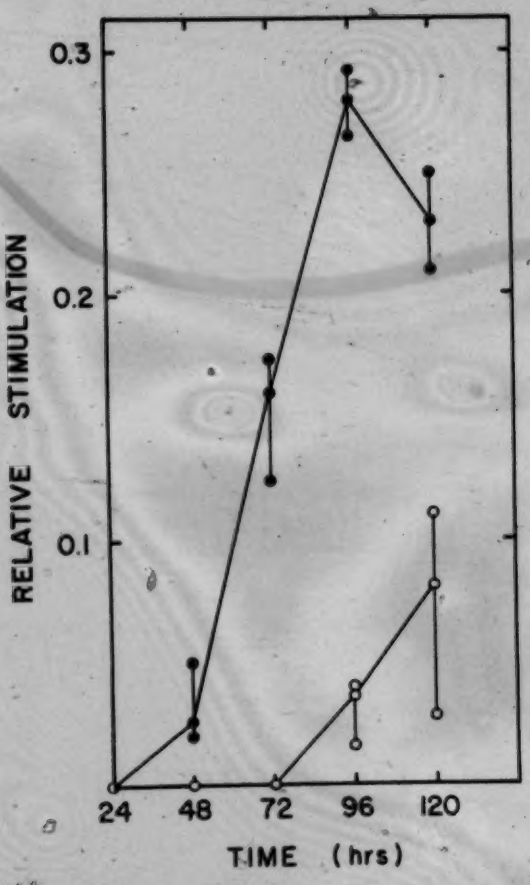

Fig. 9 - The effect of UV irradiation of stimulator cells on IL2 production in the MLR. CBA/J and gamma-irradiated DBA/2J spleen cells were co-cultured at 1 x 10^6 cells/ml each in 1 ml cultures in 16 x 100 mm glass tubes. (●)MLR; (o) stimulator DBA/2J cells were also UV-irradiated (see Materials and Methods). Three separate cultures were set up at each time point. Samples were assayed for costimulator activity at a 1/4 dilution. Results are expressed as the fraction of the maximum stimulation seen with optimal IL2.

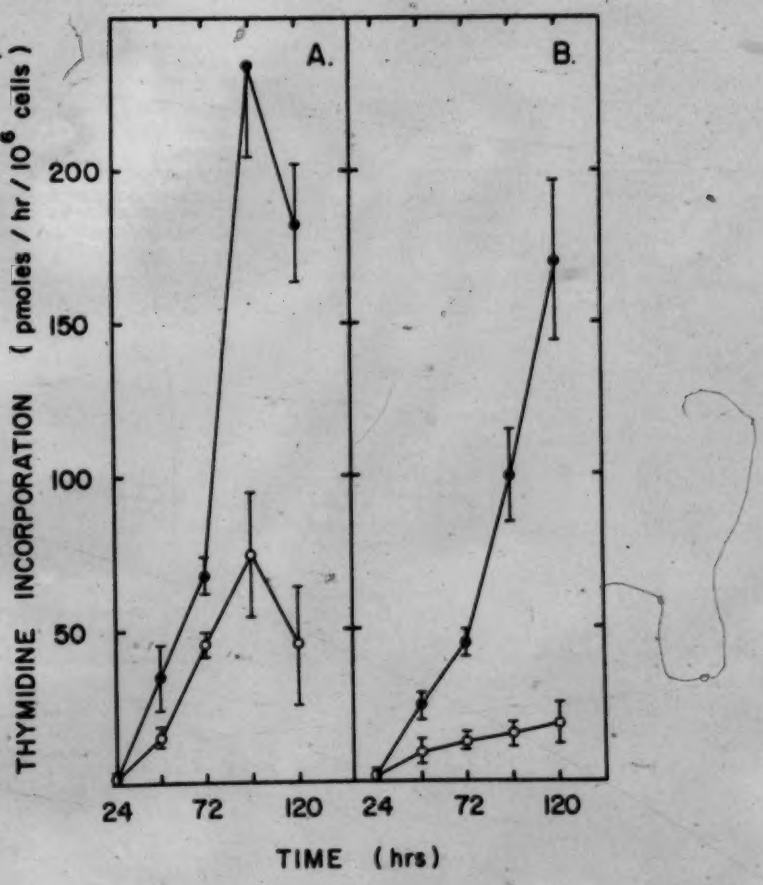

Fig. 10 - The effect of UV irradiation of the stimulator cells, and the addition of IL2, on the proliferative response of the MLR. CBA/J spleen cells and gamma-irradiated DBA/2J spleen cells were co-cultured as in Figure 9 except that microtitre plates were used instead of glass tubes. Incorporation of ^{3}H-Thd into DNA was measured in 6 replicates by a 4.5 hr pulse at the times indicated (± standard deviation). A. (o) cells alone; (●) cells plus 6 costimulator units/ml fraction 4 IL2. B. (o) stimulator DBA/2J cells UV-irradiated for 2 minutes; (●) UV-irradiated DBA/2J cells plus 6 costimulator units/ml fraction 4 IL2.

4 IL2, enhances the proliferative response seen with both UV-irradiated and normal (gamma-irradiated) stimulators. Thus, the lack of IL2 production in a MLR using UV-irradiated stimulator cells is correlated with the lack of proliferation, and this response is restored with IL2.

C. DISCUSSION

1. A Purification Protocol for IL2

A convenient, reproducible, purification protocol for IL2 was designed using the costimulator activity of IL2 to follow its biological activity. This protocol is summarized in Table 4. In the first step IL2 was precipitated with ammonium sulfate. A 50-100 fold concentration of the crude supernatants was typically obtained. Following ammonium sulfate precipitation, the IL2 was dialyzed and chromatographed over a Sephadex G-100 column to remove some inactive protein and the majority of nucleic acids and salts. This step also removed residual Con A and thus allowed IL2 activity to be studied in the absence of a mitogen known to have significant immunological effects in vitro.

Following Sephadex G-100 chromatography, fraction 3 IL2 was purified by ion-exchange chromatography on DEAE-Sephacel (Figure 2). A purification of 5-10 fold was typically observed, with quantitative recovery of activity. Large volumes of fraction 3, up to the equivalent of 10 litres of crude material, could be applied to the DEAE-Sephacel column as 80% to 90% of the protein did not bind to the resin (see Figure 2).

Fraction 4 IL2 typically had a specific activity of 1-2 costimulator units/microgram protein. IL2 purified by isoelectric focusing has a specific activity of approximately 30 costimulator units/microgram protein (Shaw *et al.* 1978b). The recovery of activity after isoelectric focusing was, however, only 10-15% in the major peak, making it impractical to routinely purify IL2 by this procedure. The ion-exchange chromatography on the other hand gave excellent recovery of activity with a 10 fold increase in specific activity. IL2 has been purifed to a level where 1 microgram of protein contains approximately 500 costimulator units (Chapter 1V). Thus, fraction 4 IL2 is less than 0.2% pure.

IL2 purified by Sephadex G-100 chromatography allowed thymocyte precursors to generate CTLs in response to alloantigen (see Chapter I,Section B4b). Both costimulator activity and helper activity for generating CTLs were present in material purified by

isoelectric focusing. It was therefore expected that these two activities would co-purify through ion-exchange chromatography, as illustrated in Figure 2. This result supports the contention that the same entity is responsible for both activities.

2. Properties of SDS-Denatured IL2

When IL2 was exposed to rigorous denaturation conditions, most, if not all, of the activity was recovered (Table 5). To assay the IL2 after SDS denaturation it was necessary to remove all traces of free SDS, so that the cells used for the assay would not be killed. This was achieved by cooling the samples to 0°C thereby precipitating out the free SDS. Any residual free SDS in the supernatant was reduced in concentration by dilution into medium containing 10% FBS prior to assay. The serum proteins in the FBS probably bind any residual SDS and thereby efficiently remove it from the sample. The recovery of IL2 activity after SDS denaturation indicates either that denatured IL2 is fully active or that it readily renatures to its active form. The various assays for IL2 all involve at least a 24 hr incubation at 37°C in the presence of medium containing 10% FBS. This may allow it to renature through the exchanging out of SDS.

Since IL2 activity was recoverable after SDS denaturation, the MW of SDS-denatured IL2 could be determined. Both gel filtration chromatography and SDS-PAGE were used. Gel filtration chromatography separates proteins on the basis of molecular size, directly yielding the Stokes radius and diffusion coefficient (Andrews 1970). A MW determination based on gel filtration chromatography assumes that the protein in question has the same molecular shape as the proteins used in the calibration. When proteins are denatured in SDS, they probably adopt a 'random coil' conformation. The reliability of MW determinations by SDS-PAGE is partly due to the uniform conformation of proteins after SDS denaturation (Shapiro *et al*. 1967; Weber and Osborn 1969). When IL2 was rigorously denatured with SDS, in the presence or absence of reducing agent, and fractionated by gel filtration chromatography, activity eluted with proteins of 16,000 MW (Figure 6). 3 activities previously associated with IL2 - costimulator activity, TCGF activity, and helper activity for CTL generation - co-purified through gel filtration chromatography (Figures 6B and 6C). This supports our previous contention that the same entity is responsible for all 3 activities (Chapter I, Section B4d). The recovery of activity after gel filtration chromatography was typically around 50% (data not shown) and

indicates that these results are representative of a majority of IL2 molecules.

The assumption of uniform molecular shape would not be valid for a protein that retained a compact structure after treatment with SDS. Fractionation of such a protein by gel filtration chromatography would yield an apparent MW smaller than the actual value. By using sedimentation velocity centrifugation in conjunction with gel filtration chromatography, the MW of a protein can be determined without any assumptions about its overall conformation (Siegel and Monty 1966). The sedimentation behaviour of SDS-denatured and DTT reduced IL2 was consistent with IL2 existing as a protein of 16,000 MW (Figure 7). If after SDS denaturation the IL2 retained a compact structure, it would have a sedimentation coeffecient comparable to proteins of greater than 31,000 MW (the MW of native IL2). Thus, the actual MW of SDS-denatured IL2 is probably 16,000, half that of native IL2.

The MW of SDS-denatured IL2 was also determined using SDS-PAGE. In different experiments, IL2 had a mobility characteristic of proteins of 30,000 (Fig. 4), 25,000 (Fig. 3) and less than 20,000 (Fig. 5) MWs. In these experiments only poor recovery of activity was obtained and it was therefore possible that these results reflected a small proportion of IL2 molecules incompletely denatured. Recently, Mochizuki *et al*. (1980b) and Granelli-Piperno *et al*. (1981) also characterised SDS-denatured IL2. Mochizuki *et al*.(1980b) fractionated IL2 by SDS-PAGE using two different discontinuous buffer systems. IL2 activity migrated with molecules of 27,000-31,000 daltons (Mochizuki *et al*. 1980b). In contrast to this, Granelli-Piperno *et al*. (1981) obtained an apparent MW for IL2 of 23,000 using SDS-PAGE. The validity of these MW values may, however, be compromised. An accurate MW determination by SDS-PAGE requires the protein of interest to have a charge-to-mass ratio equivalent to that of the proteins used as MW markers (Reynolds and Tanford 1970a,b), and to adopt a rodlike configuration (Reynolds and Tanford 1970b). Proteins in general bind constant amounts of SDS per gram (Reynolds and Tanford 1970a,b) but glycoproteins do not (Segrest *et al*. 1971; Segrest and Jackson 1972). Glycoproteins containing more than 10% carbohydrate do not bind as much SDS per gram compared with standard proteins (Segrest *et al*. 1971; Segrest and Jackson 1972). Consequently, they have a lower charge-to-mass ratio than proteins and give anomalously high MW values when characterized by SDS-PAGE. This problem is partly

overcome by increasing the acrylamide concentration of the gel thereby minimizing the effect of the charge-to-mass ratio (Segrest and Jackson, 1972). From a curve of apparent MW versus gel concentration, an asymptotic minimal MW can be obtained (Segrest and Jackson 1972). Desialated glycoproteins give even more anomalous MWs, when characterized by SDS-PAGE, due to the decrease in negative charge which, when present, compensates partly for the decreased SDS binding. The MWs of 23,000 (Granelli-Piperno *et al.* 1981) and 27,000-31,000 (Mochizuki *et al.* 1981) obtained for SDS-denatured IL2 by PAGE may be anomalously high due to the glycoprotein nature of IL2. The MW of IL2 calculated using the results from gel filtration chromatography and glycerol gradient centrifugation required no assumptions about charge-to-mass ratio. This MW value of 16,000 for SDS-denatured IL2 suggests that native IL2, which has a MW of approximately 31,000 (Shaw *et al.* 1978b) is composed of two subunits.

Mizel has observed that IL1 activity could also be recovered after SDS-denaturation (Mizel 1979), and that SDS-denatured IL1 had a MW of 12,000. A value of 18,500 was obtained for SDS-denatured IL1 using gel filtration chromatography (data not shown). The MW of native IL1 is 12,000-16,000 (Mizel 1979), and thus IL1 is composed of a single polypeptide chain.

The apparent MW of both rat and human IL2, as determined by gel filtration chromatography was 15,000 (Gillis *et al.* 1980b). All 3 species of IL2 studied so far - rat, human, and now mouse - can all exist as polypeptide chains of 15,000-16,000 MW.

The Role of IL2 in the MLR to UV-Irradiated Stimulator Cells

One of the major functions ascribed to IL2 is to confer immunogenicity to T cell responses (Chapter 1, Section C2). When spleen cells are stimulated in a MLR they proliferate. If, however, the stimulators are UV-irradiated no proliferration occurs. These UV-irradiated stimulators do express recognizable antiren on their surfaces yet they are no longer immunogeneic (see Table 2). Figure 10 illustrates that UV irradiation almost completely abrogates proliferation in the MLR. This response is restored when exogenous IL2 is added. UV-irradiated stimulators are also unable to elicit IL2 production (Figure 9), thus suggesting a correlation between the lack of proliferation and the inability to stimulate IL2 production. Similar results have been obtained by Okada *et al.* (1978) and Paetkau *et al.* (1980) in the CTL response against UV-irradiated stimulator cells. A correlation between a

lack of IL2 production and a decreased level of cytotoxicity was observed. That the missing 'signal' with UV-irradiated stimulators is IL2 is suggested by the results in Figure 8 and those of Paetkau *et al*. (1980a) and Kern *et al*. (1981). This suggests that the role of IL2 is to turn an antigenic signal into an immunogenic one. IL2 has been shown to convert antigenicity into immunogenicity in several other situations. These are described in Chapter 1, Section C2, and argue for a physiological role for IL2 in immune activation.

IV. STUDIES ON IL2 PRODUCED FROM EL4 LYMPHOMA CELLS

A. INTRODUCTION

In the previous chapter, IL2 was characterized by several purification procedures. Several properties of SDS-denatured IL2 were also described. These studies were based on the ability of IL2 to stimulate a particular immunological response. Homogeneous preparations of IL2 were unavailable for study due to the paucity of material produced by Con A-stimulated spleen cells. Recent estimates suggest that IL2 is active at 10^{-13} M (see Discussion). Based on the amount of IL2 activity produced by Con A-stimulated spleen cells (typically 10-50 units/ml), only nanogram amounts of IL2 protein were produced per ml. Total protein was in the range of 0.1 to 1.0 mg/ml, thus necessitating a purification of over 10^6 fold to yield homogeneous material.

An approach to obtaining homogeneous material is to use cells which produce a much higher titer of IL2 than do spleen cells. S. Gillis, J. Watson and coworkers (Gillis *et al.* 1980a) screened over 40 established T cell leukemias and lymphomas for IL2 production. One, LBRM-33, when stimulated with the T cell mitogen PHA, produced greater than 1000 fold the amount of IL2, on a per cell basis, than did Con A-stimulated spleen cells. This cell line provides an excellent source of IL2 for biological experiments and a clonal source of IL2 for biochemical characterization.

Another T cell lymphoma shown to produce an extremely high titre of IL2 is a variant of EL4 discovered by Dr. J. Farrar (National Institute of Health, Bethesda, Maryland). These EL4 lymphoma cells, when stimulated with PMA produced 500 fold more IL2 than did spleen cells (Farrar, J.J. *et al.* 1980a). This cell line was kindly provided to us by Dr. Farrar. The experiments in this chapter describe conditions for IL2 production from the EL4 lymphoma cells. In addition, the IL2 produced was fractionated by the chromatographic procedures established in Chapter III. The preliminary characterization of the IL2 produced by the EL4 lymphoma cells has been reported by Dr. Farrar (Farrar, J.J. *et al.* 1980a).

B. RESULTS

1. TCGF Assay

The majority of results presented in this chapter are based on the TCGF activity of IL2. TCGF, and the assay system for it, were first described by Gillis, Smith and coworkers (Gillis *et al*. 1978b).

The TCGF assay was performed as described in Materials and Methods. A continuous, cloned mouse T cell line, MTL 2.8.1 (Bleackley *et al*. 1982) was used. Briefly, cells were cultured in flat bottom microtitre plates at a density of 1×10^4 cells/well in 0.2 ml of RHFM containing the sample of IL2 to be assayed. After 18–24 hr incubation the cells were exposed for 4–6 hr to ^{125}I–UdR and then harvested. Results are expressed as either (1) % maximum stimulation at a particular dilution with maximum stimulation determined at an optimal concentration of IL2; or (2) units of TCGF activity, based on the concentration of IL2 required to give 1/3 the maximum response, as described for the costimulator assay (Figure 1).

The results obtained with the TCGF assay were similar to those of the costimulator assay. Similar dilution curves to the one depicted in Figure 1 were routinely obtained (data not shown) and were used to calculate TCGF activity. The TCGF assay is approximately 4 fold more sensitive to IL2 than is the costimulator assay and can be made even more sensitive by decreasing the number of cells used (C. Havele, unpublished observations).

2. Production of IL2

The conditions for IL2 production from the EL4 lymphoma cells were determined using either cells as provided to us by Dr. Farrar, or obtained from cloning the EL4 lymphoma line (see below). Table 6 illustrates that EL4 lymphoma cells stimulated with PMA produce IL2. The optimal concentration of PMA was 10 ng/ml. Con A stimulation of these cells, at concentrations mitogenic for murine spleen cells, neither stimulated IL2 production itself nor influenced the amount of IL2 produced upon PMA stimulation. 12×10^6 Con A–stimulated spleen cells produced 10–50 costimulator units of IL2; 1×10^6 EL4 lymphoma cells produced 1,000–5,000 TCGF units of IL2. On a per cell basis, with the TCGF assay 4 fold more sensitive than the costimulator assay, this represents an average 250 fold increase in the amount of IL2 produced.

TABLE 6

EFFECT OF CON A AND PMA STIMULATION ON IL2 PRODUCTION[a]

EL4 Cells Stimulated with:	TCGF Activity[b] (units/ml)
-	<10
1 ng/ml PMA	20
10 ng/ml PMA	1,200
50 ng/ml PMA	800
3 micrograms/ml Con A	<10
3 micrograms/ml Con A + 10 ng/ml PMA	1,200
6 micrograms/ml Con A	<10
6 micrograms/ml Con A + 10 ng/ml PMA	1,200

[a] EL4 · E1 cells were grown in the ascities form in mice before use. 4 ml cultures were set up in plastic T flasks in RH medium containing 4% HS. Cells were cultured at a density of 1 x 10^6 cells/ml and stimulated with the indicated reagent for 24 hr. Cell-free supernatants were assayed for TCGF activity.

[b] Results are expressed as the average number of units in duplicate samples.

A time course for IL2 production from the EL4 lymphoma cells is illustrated in Figure 11. The EL4 cells were grown in the ascites form in mice (see Materials and Methods), washed twice, and then cultured in RH medium containing 5% HS. The cells were stimulated with 10 ng/ml PMA for the times indicated. Optimal production of IL2 occured between 9 and 18 hr after stimulation with little or no change in the amount of IL2 present after up to 72 hr of culture.

Experiments which studied the effect of the medium used during IL2 production indicated that the presence of either HS or FBS in the culture medium increased IL2 production (Table 7). Between 2 and 25 fold greater levels of IL2 were produced by EL4 cells stimulated in medium containing serum. Optimal serum was a combination of 4% HS, 1% FBS (Table 7). Further experiments demonstrated that 4% HS alone gave comparable results (data not shown).

The conditions used to grow the EL4 cells prior to stimulation also had an effect on IL2 production (Table 7). Cells grown in vitro for several weeks in medium containing 5% HS produced significantly more IL2 than those cultured in vitro in RH medium containing 10% FBS (Table 7, Experiment 1). This increase in IL2 was observed in all combinations of media present during PMA stimulation. The most efficient production of IL2 occured when cells were used immediately after being grown in the ascites form (Table 7, Experiment 2). That the increase in the amount of IL2 activity produced actually represents IL2 and not an effect of different sera, or other components present in crude supernatants, on the TCGF assay is demonstrated in Table 8. The difference in the apparent level of IL2 activity produced was maintained through various stages of purification, including gel filtration chromatography and ion-exchange chromatography (Table 8).

These experiments established the conditions for generating high titers of IL2 from the EL4 lymphoma cells. The cells were grown in the ascites form in mice and harvested from the peritoneal cavity prior to use in IL2 production. They were washed twice, cultured at a density of 1×10^6 cells/ml in RH medium containing 4% HS and stimulated for 24 hr with 10 ng/ml PMA.

3. Cloning of the EL4 Lymphoma Cell Line

EL4 lymphoma cells were cloned by limiting dilution. Cells were cultured in micro-titre plates at an average 0.25 cells/well. Most wells in which cell growth occurred thus

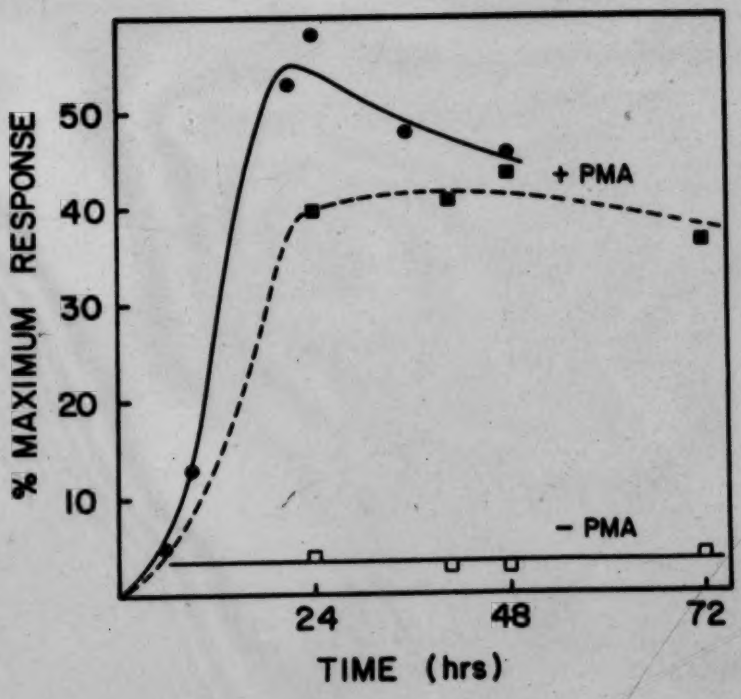

Fig. 11 - Time course of IL2 production by EL4 lymphoma cells. EL4 lymphoma cells were grown as ascites in C57B1/6J mice as described in Materials and Methods. The cells were cultured at 1 x 10^6/ml in RHM medium containing 5% HS and 10 ng/ml PMA. At the times indicated, the 4 ml samples were harvested and dialyzed. They were then assayed for costimulator activity at a 1/100 dilution. The results from two separate experiments are shown (● , ■), as well as a control, (□), which consisted of EL4 lymphoma cells cultured in the absence of PMA to which 10 ng/ml PMA was added to the cell-free supernatant prior to dialysis.

TABLE 7

PRODUCTION OF IL2 BY EL4 LYMPHOMA CELLS:
EFFECT OF SERA-CONTAINING MEDIA AND IN VITRO CULTURE ON IL2 PRODUCTION

Cells Stimulated in vitro in Medium Containing:	Experiment 1 (a) Costimulator Activity (c) (% Maximal Stimulation)		Experiment 2 (b) TCGF Activity (units/ml)	
	Cells cultured in vitro in 5% HS	Cells cultured in vitro in 5% FBS	Ascites Cells	Cells Cultured in vitro in 4% HS
No Serum	13.8	6.6	300	30
1% FBS	15.6	5.6	700	400
5% FBS	32.7	14.1	N.D.	N.D.
10% FBS	N.D. (d)	N.D.	600	300
1% HS	12.3	5.7	800	300
2% HS	N.D.	N.D.	800	350
5% HS	43.3	14.3	1,200	500
1% HS, 4% FBS	47.2	15.8	N.D.	N.D.
4% HS, 1% FBS	43.8	10.4	1,600	800

(a) EL4 cells were cultured for several weeks in RH medium containing either 5% HS or 5% FBS. The cells were washed 2 times in RH medium and resuspended at a concentration of 1×10^6 cells/ml in the appropriate media. Duplicate 4 ml cultures were stimulated with 10 ng/ml PMA for 24 hours and the cell-free supernatant assayed for costimulator activity.

(b) Cells of clone EL4-E1 were grown as ascites in C57Bl/6J mice as described in Materials and Methods or grown in vitro in RH medium containing 4% HS. The cells were washed and cultured as described above. Cell-free supernatants were assayed for TCGF activity.

(c) Costimulator activity was assayed for at a dilution of 1/100.

(d) Not determined.

TABLE 8

IL2 PRODUCTION[a] FROM EL4 LYMPHOMA CELLS STIMULATED IN VITRO IN MEDIA CONTAINING EITHER HS OR FBS: RECOVERY OF IL2 AFTER VARIOUS STAGES OF PURIFICATION[b]

Stage	Purification Procedure	TCGF Activity (Total units) EL4 cells stimulated in vitro in medium containing	
		10% FBS	5% HS
Crude	-	9,700	35,000
Fract 2	Ammonium sulfate precipitation	7,400	43,000
Fract 3	Sephadex G-100 chromatography	4,100	15,700
Fract 4	DEAE-Sephacel chromatography	1,700	8,000

(a) EL4 lymphoma cells were grown as ascites, cultured at 1×10^6 cells/ml in the medium indicated, and stimulated for 24 hr. with 10 ng/ml PMA. The crude supernatants were 280 ml each.

(b) See text for purification procedure.

represented clones. These clones were tested for their ability to produce IL2. The cloning and subsequent testing of the clones for IL2 production was done by Dr. C. Havele.

A correlation between the growth characteristics of the clones and their ability to produce IL2 was observed. When stimulated with PMA, the clones which continued to proliferate did not produce IL2 whereas those that stopped proliferating did (C. Havele, unpublished observations).

Of 24 randomly selected clones, 14 (approximately 60%) were shown to produce high levels of IL2 in response to PMA. 6 of these clones were grown in the ascites form in mice and then tested for IL2 production. One clone, designated EL4.E1, produced 4 times more IL2 than did the uncloned line and was therefore used in all subsequent experiments.

4. Purification of IL2

The techniques established for the partial purification of IL2 produced from Con A–stimulated spleen cells were applied to the IL2 produced from the EL4 lymphoma cells. A major difference in the production of IL2 from these two sources is that the latter source of IL2 is produced in medium containing 5% HS, whereas the IL2 from spleen cells is produced in serum–free medium. As a result, the purification of IL2 from the EL4 lymphoma cells requires handling much more protein.

EL4–produced IL2 was ammonium sulfate precipitated at 80% saturation (see Materials and Methods). The efficacy of this procedure was shown by the observations that (1) quantitative recovery of activity was obtained after ammonium sulfate precipitation and (2) precipitation of material previously precipitated (and then dialyzed) had no effect on the amount of IL2 recovered (data not shown). Thus, ammonium sulfate precipitated all of the IL2 with no adverse effect.

Following ammonium sulfate precipitation, the IL2 was dialyzed (fraction 2) and concentrated by lyophilization. A total concentration of 50–100 fold was achieved by these two procedures. The concentrated fraction 2 IL2 was then chromatographed over a Sephadex G–100 column. TCGF activity eluted with proteins of apparent MW around 30,000.

Following Sephadex G–100 chromatography, the IL2 (fraction 3) was further purified by hydrophobic interaction chromatography on Phenyl–Sepharose, as illustrated in Figure 12 (see also Hilfiker *et al.* 1981). The IL2 was applied in high salt (2.5 M NaCl) which

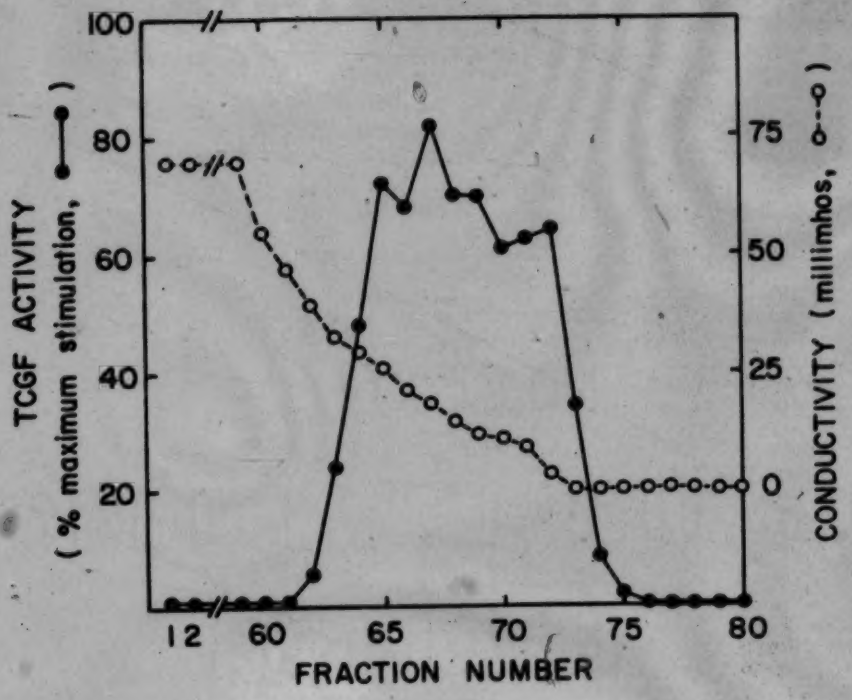

Fig. 12 - Phenyl-Sepharose chromatography of IL2. Fraction 3 IL2 was chromatographed by Phenyl-Sepharose as described in Materials and Methods. Fractions were assayed for TCGF activity at a dilution of 1/2000. The gradient was monitored by conductivity.

favors hydrophobic interactions, and activity eluted with a gradient from 2.5 M NaCl to 50% ethylene glycol. 90% of the applied activity was eluted with 10% ethylene glycol, which also eluted the majority of protein present in the sample (data not shown). The Phenyl-Sepharose fractions were also assayed for colony stimulating factor activity for granulocytes and macrophages. Colony stimulating factor promotes the differentiation of macrophages and granulocytes from bone marrow precursors (reviewed in Burgess *et al.* 1978). The colony stimulating factor assay was performed by M. Wong and Dr. K. -C. Lee. Under the conditions used, the colony stimulating factor did not bind to the Phenyl-Sepharose column and was therefore completely seperated from IL2 (data not shown, Hilfiker *et al.* 1981).

Phenyl-Sepharose-purified IL2 (fraction 4) was dialyzed against buffer A (see Materials and Methods) and then fractionated by DEAE-Sephacel chromatography. 90% of the applied activity eluted at a salt concentration of 0.12 to 0.14 M NaCl. This IL2 is referred to as fraction 5. A significant amount of the TCGF activity did not however bind to the DEAE-Sephacel resin. Typically, about 10% was observed in the DEAE-Sephacel run-through fraction. This 'DEAE run-through' material stimulated the TCGF assay to only 30% of the maximum response seen with optimal IL2. As illustrated in Figure 13, the DEAE run-through material does not stimulate the assay in the same manner as does fraction 5 (and all other preparations of) IL2. In addition, both thymocyte proliferation in response to Con A (the costimulator assay), and the proliferation of an established natural killer cell line (Dennert 1980), were also stimulated to only 30% of the maximun response with the DEAE run-through material (data not shown; G. Dennert, personal communication) This material behaved as a protein of 30,000 MW through gel filtration chromatography on Sephadex G-100 and had a higher isoelectric point than IL2 (as determined by chromatofocusing, see below). Preliminary experiments with the DEAE run-through material indicate that lymphoid cells can be grown continually in culture in its presence (C. Havele, unpublished observations). These lymphoid cells are morphologically different from the cells which are grown in IL2. This DEAE run-through material is similiar to the lymphokine recently described by Ihle and coworkers (Ihle *et al.* 1981; Hapel *et al.* 1981) and referred to as Interleukin 3 (IL3, see Discussion).

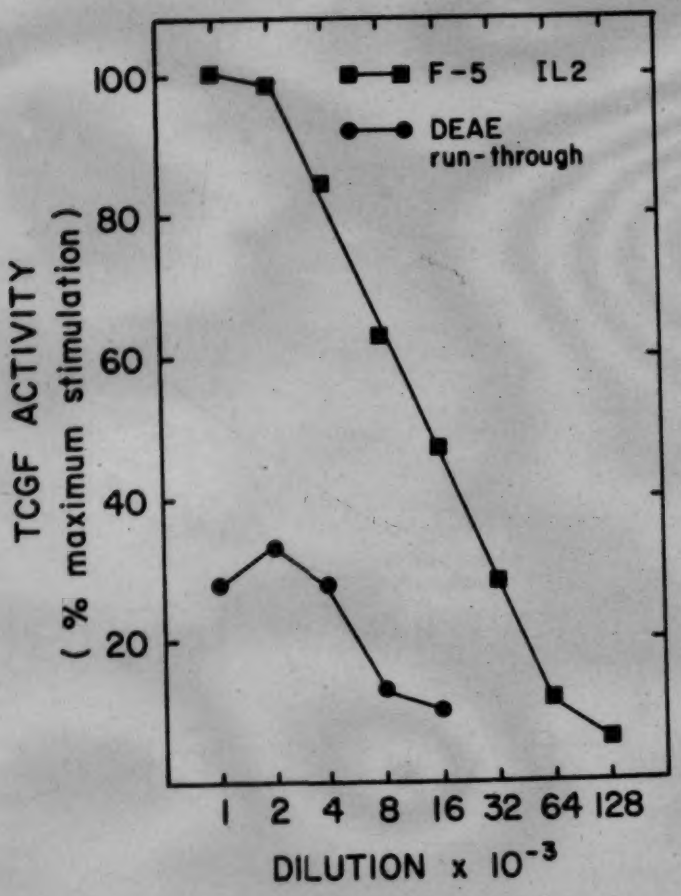

Figure 13 - Titration of IL2 after DEAE-Sephacel chromatography. Fraction 4 IL2 was chromatographed on DEAE-Sephacel as described in Materials and Methods. The material which eluted in the run-through fraction (DEAE run-through, see text), and that which eluted at a salt concentration of approximately 0.15 M (F-5) were titrated in the TCGF assay.

Fraction 5 IL2 was further purified by a second fractionation on Sephadex G-100 (Figure 14), to further increase specific activity. From its behaviour on this column, most of the contaminating protein was probably horse serum albumin, the major component of the horse serum used during IL2 production.

A summary of the results is given in Table 9. Overall, close to 30% of the activity was recovered, with an increase in specific activity of approximately 20 fold. Fraction 6 IL2 had a specific activity of 10 TCGF units/microgram protein. The most effective purification step was the final fractionation over Sephadex G-100. Over 80% of the activity in fraction 5 IL2 was recovered, with an increase in specific activity of over 10 fold.

The fractions obtained from this purification procedure were assayed for costimulator activity in addition to TCGF activity. The recovery of costimulator activity was essentially identical to that of TCGF activity, supporting the conclusion that both assays measure the same entity.

Fraction 6 IL2 was further characterized and purified by chromatofocusing. Chromatofocusing separates proteins on the basis of their isoelectric point (see Materials and Methods). It involves binding the protein of interest to an anion exchange resin at high pH, and subsequently eluting the protein from the resin with ampholytes titrated to low pH. Proteins elute from the column in buffer close to their isoelectric points. When fraction 6 IL2 was fractionated by chromatofocusing (Figure 15), it was resolved into 9 peaks of activity ranging in isoelectric point from 4.6 to 3.4. A peak of IL2 activity also eluted from the column when the resin was subsequently washed with 1 M NaCl. The majority of the protein present in the fraction 6 IL2 eluted at a pI of between 4.4 and 4.5. This is consistent with this protein being horse serum albumin. Several different preparations of IL2 gave essentially identical results to those illustrated in Figure 15.

The specific activities of the various isoelectric forms of IL2 are given in Table 10. The fraction 6 IL2 applied to the chromatofocusing column had a specific activity of 10 units/microgram protein, and that eluted with 1 M NaCl had 2,000 units/microgram – a 200 fold increase. This IL2 typically represented between 15-20% of the IL2 applied (see Table 10). Total recovery of activity was usually 70-80%.

When re-fractionated by chromatofocusing, the IL2 which eluted with the 1 M NaCl wash again eluted with this high salt wash (data not shown). The most likely

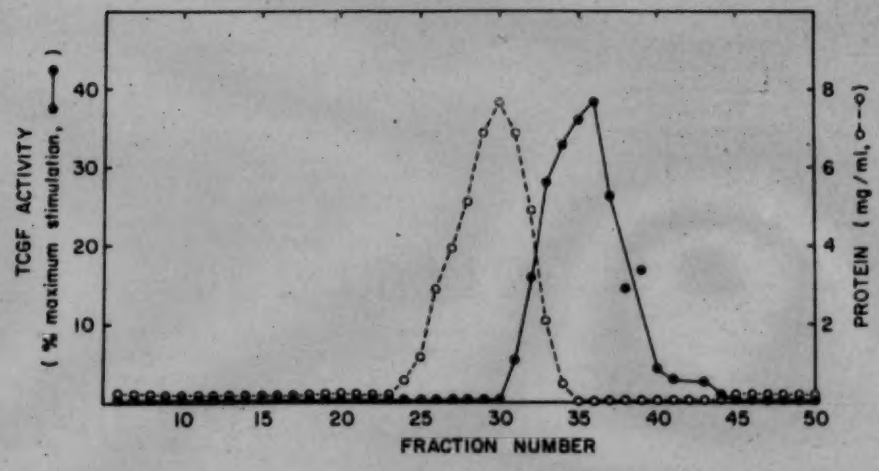

Figure 14 - Sephadex G-100 chromatography of fraction 5 IL2. Fraction 5 IL2 (see Table 9) was lyophilized, resuspended in a final volume of 8 ml and applied to a Sephadex G-100 column, as described in Materials and Methods. Each fraction (7.9 ml) was assayed for TCGF activity at 1/1000 dilution. The amount of protein in each sample was determined by the Coomassie Blue dye method.

TABLE 9

PURIFICATION OF IL2 DERIVED FROM EL4 LYMPHOMA CELLS[a]

Stage	Purification Procedure	Vol. (ml)	Protein (mg)	Activity (units)	SP. Activity (units/mg protein)	Yield (%)
Crude	-	2,500	5,000	2.5×10^6	500	-
Fract 2	Ammonium sulfate precipitation	355	5,000	2.8×10^6	560	112
Fract 3	Sephadex G-100 chromatography	176	2,000	1.7×10^6	850	68
Fract 4	Phenyl-Sepharose chromatography	250	1,200	1.5×10^6	1,250	60
Fract 5	DEAE-Sephacel chromatography	60	1,100	0.84×10^6	760	34
Fract 6	2nd Sephadex G-100 chromatography	8.8	70	0.70×10^6	10,000	28

(a) see text for explanation

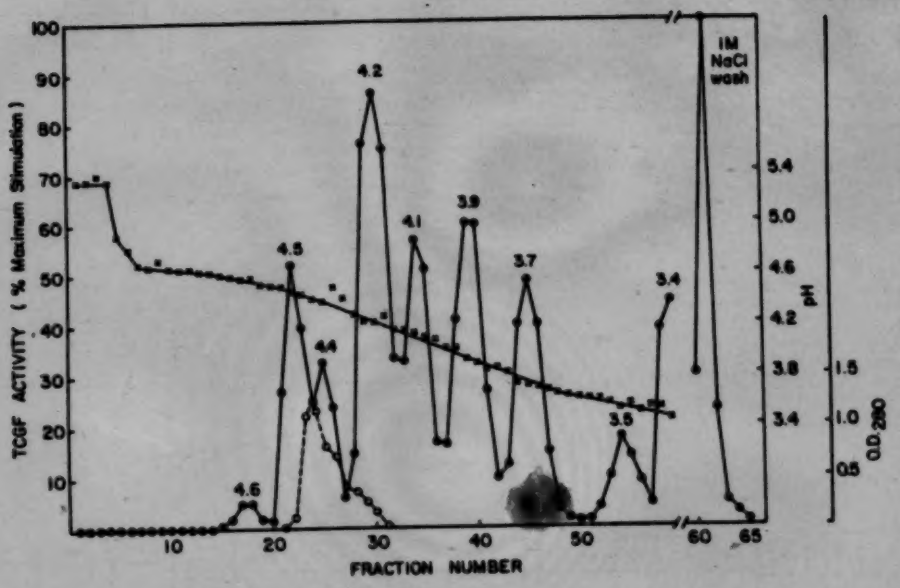

Figure 15 – Chromatofocusing of fraction 6 IL2. Fraction 6 IL2 (see Table 9) was characterized by chromatofocusing as described in Materials and Methods. Fractions (4.1 ml) were assayed for TCGF activity at 1/500 dilution (●). The number above each peak of activity represents the isoelectric point of that peak as determined from the pH of the most active fraction. The OD_{280} of each fraction was also determined (o).

TABLE 10

PURIFICATION OF IL2 BY CHROMATOFOCUSING: RECOVERIES AND SPECIFIC ACTIVITIES[a]

Sample	Vol. (ml)	Protein (micrograms)	Activity (units x 10^{-3})	Sp. Activity (units/microgram protein)	Yield %
Fract 6	5	40,000	400	10	-
Chromatofocusing Species					
pI 4.6	8	8	.08	10	.02
4.5	16	9,000	19.2	2.1	4.8
4.4	8	6,400	6.4	1.0	1.6
4.2	20	5,000	80.0	16	20
4.1	16	464	16.0	34	4
3.9	20	40	16.0	400	4
3.7	20	100	8.0	80	2
3.5	16	32	0.8	25	0.2
3.4	12	275	12.0	43	3.0
1 M NaCl Wash	8	32	64.0	2,000	16.0

(a) fraction 6 IL2 was fractionated by chromatofocusing as detailed in Figure 16.

(b) the chromatofocusing species are those indicated in Figure 16.

explanation is that this represents IL2 with pI less than 3.4, which would remain bound to the anion exchange resin throughout the elution protocol. Indeed, when IL2 was fractionated by chromatofocusing using ampholytes titrated to pH 3.0, a species of IL2 eluted with a pI of 3.1, and a significant decrease in the amount of IL2 in the 1 M NaCl wash was observed (data not shown).

This 1 M NaCl wash material is not homogeneous. Analysis by SDS-PAGE showed a major band of protein in the MW range of 70,000, and several other protein bands around 20,000 MW (data not shown). On the basis that the TCGF assay can detect 0.1 unit/ml IL2, IL2 is active at less than 3×10^{-12} M (see Discussion).

Figure 16 shows a comparison of the chromatofocusing profiles obtained with IL2 from spleen cells and from EL4 lymphoma cells. The TCGF activity of each fraction is expressed as a function of the pH of that fraction. The IL2 produced from Con A-stimulated spleen cells is very similar to that produced from the EL4 lymphoma cells. Neither preparation contained IL2 with isoelectric points greater than 4.6. Both preparations contained IL2 which had pI values of 4.5, 4.4, 4.2, 4.1 and 3.7, and IL2 which eluted with the 1 M NaCl wash.

The fractions obtained from chromatofocusing were also assayed for costimulator activity and an identical profile of activity was obtained (data not shown), supporting the conclusion that both assays measure the same entity.

When fraction 6 IL2 was denatured with SDS (see Materials and Methods) and fractionated by gel filtration chromatography, it eluted with proteins of 16,000 MW (Figure 17). The profile illustrated in Figure 17 shows that the majority of protein remaining in fraction 6 material does not have IL2 activity. This protein, with a MW of approximately 67,000, is probably horse serum albumin.

C. DISCUSSION

1. TCGF Assay

The experiments described in this chapter deal with the biochemical characterization of IL2 derived from PMA-stimulated EL4 lymphoma cells. Throughout these experiments, the behaviour of IL2 was mainly determined by its TCGF activity. The major advantage of the TCGF assay over the costimulator assay is that the T cells used are

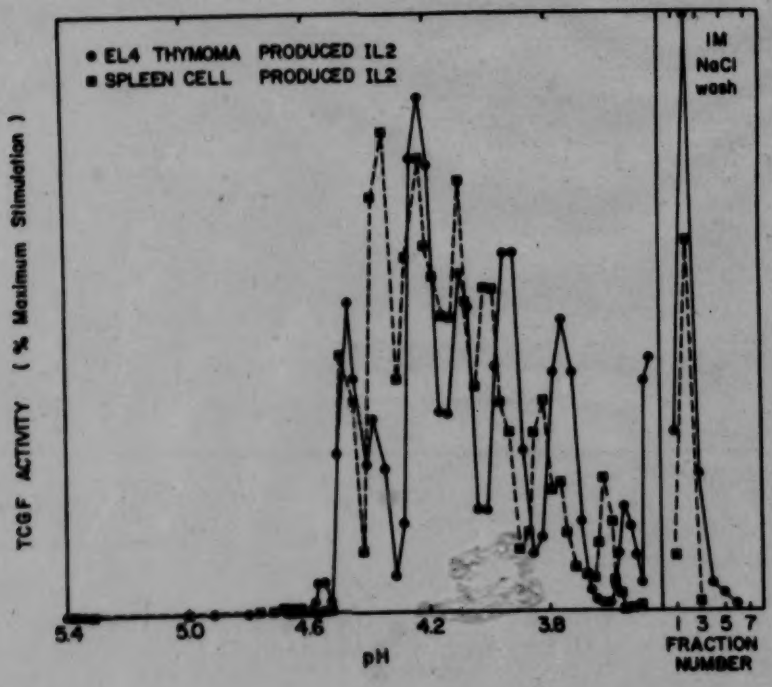

Figure 16 - Comparison of IL2 produced by Con A-stimulated spleen cells and PMA-stimulated EL4 lymphoma cells: Chromatofocusing profiles. Spleen cell-produced IL2 was purified to fraction 4 (see Table 4) and characterized by chromatofocusing. Fractions were assayed for TCGF activity at a 1/100 dilution. The IL2 from PMA-stimulated EL4 lymphoma cells was purified to fraction 6 and similarly characterized by chromatofocusing (Figure 15). The TCGF activity of each fraction is plotted against the pH of that fraction, thereby allowing a direct comparison of the results obtained with these two preparations of IL2.

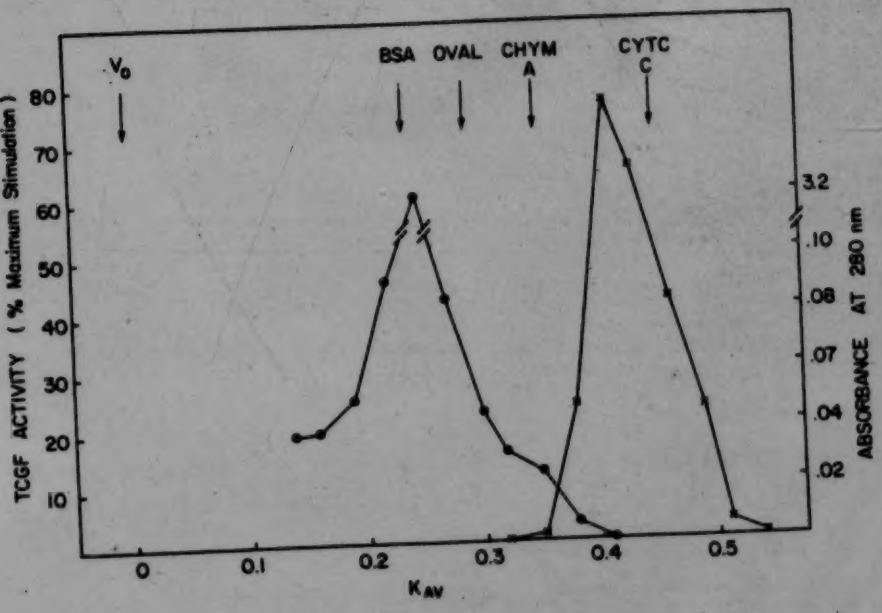

Figure 17 - Gel filtration chromatography of EL4 lymphomaproduced IL2 after SDS denaturation. Fraction 6 IL2 (Table 9) was denatured with 1% SDS and 10 mM DTT for 10 min at 70°C. The sample was then chromatographed as described in Figure 6. Fractions were assayed for TCGF activity (✕) at a 1/1000 dilution after precipitation of free SDS. The elution positions of the MW markers are indicated by arrows, markers as in Figure 3. The OD_{280} was also determined (●).

clonal and are responsive only to mediators which directly stimulate T cell proliferation. Throughout the fractionations the TCGF and costimulator assays gave identical results, indicating that they measure the same entity. Both involve the proliferation of activated T cells – in the TCGF assay the first activator is alloantigen and in the costimulator assay, it is Con A. It is therefore not surprising that a single entity stimulates both assays.

2. Production of IL2 from EL4 Lymphoma Cells

High titers of IL2 were produced from the EL4 lymphoma cells when they were grown in the ascites form in mice, cultured at a density of 1×10^6 cells/ml in RH medium containing 4% HS, and stimulated with 10 ng/ml PMA for 24 hr. The results which established these conditions suggested that there was an inverse correlation between the proliferation of these cells and their ability to produce IL2. The EL4 lymphoma cells grew better in medium containing FBS than they did in medium containing HS. The cells grown in FBS produced significantly less IL2 than those grown in medium containing HS (Table 7, Experiment 1). Cells harvested directly from the peritoneal cavity of mice were under even less favorable conditions for proliferation due to their extremely high density (greater than 10^8 cells/ml). These cells were the most efficient in producing IL2 (Table 7, Experiment 2). Further support for the inverse relationship between proliferation and IL2 production were the results comparing IL2 production in different media. EL4 lymphoma cells stimulated in medium containing FBS produced less IL2 than did cells stimulated in medium containing HS – the poorer growth medium. Furthermore, only those EL4 clones whose proliferation was inhibited by PMA produced IL2 (C. Havele, unpublished observations). That is, those clones which continued to grow in the presence of PMA were unable to elaborate IL2. Although other interpretations of these results are possible, they establish conditions for the production of high titres of IL2 – typically greater than 250 times the amount produced from Con A–stimulated spleen cells.

The mechanism by which PMA stimulates the EL4 lymphoma cells to produce IL2 is unclear. Farrar and coworkers (Farrar, J.J., *et al.* 1980a) have argued that the EL4 lymphoma cells constitutively produce IL2 during the G_1 phase of their cell cycle and subsequently utilize the IL2 for their own proliferation during S phase. These investigators proposed that significant accumulation of IL2 in the culture medium occurs only after PMA has inhibited the EL4 cells from leaving G_1. Data supporting this argument, and showing a

relationship between cell cycle and recovery of IL2 activity, has recently been reported (Stadler *et al.*, 1981). However, we have obtained evidence that the synthesis of IL2 probably does increase in response to PMA. Messenger RNA (mRNA) for IL2 has been isolated from EL4 lymphoma cells and translated in frog oocytes into biologically active material. Only EL4 lymphoma cells stimulated with PMA contained detectable levels of translatable mRNA for IL2. This suggests that PMA induces a differentiative event in these cells such that they produce high levels of IL2 mRNA (and subsequently IL2) and stop proliferating.

Several other clonal sources of IL2 have been described. As previously mentioned, Gillis *et al.* (1980a) have reported the production of high titers of IL2 from LBRM-33 cells stimulated with either PHA or Con A. The LBRM-33 cell line is clearly different from the EL4 lymphoma cell line. The EL4 lymphoma cells were derived from a different strain of mice and do not produce IL2 in response to Con A stimulation (Table 6, Farrar, J.J. *et al.* 1980a), as do the LBRM-33 cells. The LBRM-33 cells do, however, respond to PMA stimulation, but only in the presence of sub-optimal amounts of PHA or Con A (Gillis *et al.* 1980a). The IL2 from the EL4 lymphoma cells, and that from the LBRM-33 cells, are biochemically indistinguishable from the IL2 produced by Con A-stimulated spleen cells (discussed below, and Farrar, J.J. *et al.* 1980a; Mochizuki *et al.* 1980a).

Two groups of investigators have recently reported the generation of T cell hybridomas which produce IL2 (Harwell *et al.* 1980; Schrader and Clark-Lewis 1981). Both hybridomas produced IL2 in response to Con A stimulation. Nabel *et al.* (1981b) have recently characterized a cloned helper T cell line (Lyt 1^+, 2^-) which produces IL2. The production and release of IL2 occurred in the absence of any apparent stimulation.

The majority of the cloned T cell sources of IL2 described above have been shown to produce lymphokines other than IL2. Several have been shown to produce colony stimulating factor, including the EL4 lymphoma cell line (Hilfiker *et al.* 1981), the helper T cell line of Nabel *et al.* (1981b) and the hybridoma of Schrader and Clark-Lewis (1981) and Schrader *et al.* (1980). The other cloned T cell sources of IL2 have not been tested for colony stimulating factor production. The EL4 lymphoma cell line, and probably the hybridoma of Harwell *et al.* (1980), produce 'B cell growth factor' in addition to IL2 (J. Farrar, personal communication). Thus, even though IL2 is available from a homogeneous

population of T cells, these cells produce lymphokines other than IL2. It therefore remains important to perform experiments with preparations of IL2 which have been at least partially purified.

3. Purification of IL2

The purification protocol for the IL2 produced from EL4 lymphoma cells was based on the techniques established for the IL2 produced from Con A-stimulated spleen cells. This protocol is summarized in Table 9. The IL2 derived from EL4 lymphoma cells behaved similarly to that derived from spleen cells stimulated with Con A, having (1) the same molecular radius (as determined by gel filtration), (2) the same net charge (as determined by ion-exchange chromatography), and (3) the same heterogeneity in isoelectric points (as determined by chromatofocusing, Figure 16). In addition, this IL2 behaved similarly to spleen cell-produced IL2 through hydrophobic interaction chromatography on Phenyl-Sepharose, and gel filtration chromatography after SDS denaturation.

Overall, the purification protocol to fraction 6 resulted in a 20 fold increase in specific activity with approximately 30% recovery of activity (Table 9). The greatest increase in specific activity occured in the second fractionation on Sephadex G-100, which was performed under conditions favoring high resolution. This fractionation alone resulted in a greater than 10 fold increase in specific activity. Up until this step only a limited increase in specific activity was observed (Table 9). This is mainly due to the fact that horse serum albumin, the major contaminating protein in these preparations, is not completely removed up to this point. The increase in specific activity observed up untill the second G-100 chromatography therefore does not represent the efficacy of the various purification procedures in removing contaminating lymphocyte proteins. The Phenyl-Sepharose chromatography, for example, gave only a moderate increase in specific activity (approximately 1.5 fold) but this procedure completely resolved IL2 from colony stimulating factor (data not shown, Hilfiker *et al.*, 1981). The DEAE-Sephacel chromatography resulted in a decrease in specific activity, yet it separated IL2 from a lymphokine with different biological properties (see below). These procedures are therefore important in purifying and characterizing IL2, as was seen with spleen cell-produced IL2 from serum-free media (Chapter III).

The lymphokine which eluted in the DEAE-Sephacel run-through fraction is particularly interesting. This material is different from IL2 as indicated by ion-exchange chromatography and chromatofocusing. When fractionated by chromatofocusing this lymphokine eluted with a pI value higher than those associated with IL2 (data not shown). There was no detectable TCGF activity in the region where IL2 would be expected to elute, indicating that the DEAE run-through material did not contain IL2. The ability of this material to stimulate the TCGF assay to only 30% of the maximum response is difficult to explain. The T cells used in the TCGF assay are all derived from a single cell and thus should behave identically. It appears that only a proportion of these cells (3 out of 10) are able to respond. Alternatively, all of the cells may be responding to this lymphokine but differently than they do to IL2, such that they do not incorporate ^{125}I-UdR as efficiently. Evidence for this second alternative has recently been obtained (C. Havele, unpublished observations). The stimulation to only 30% of the maximum response was also observed in the costimulator assay (data not shown) and in the proliferation of a continuous natural killer cell line (G. Dennert, personal communication), and thus is not a unique property of the MTL 2.8.1 cell line.

A possible identity for this DEAE run-through material is the lymphokine recently described by Ihle and coworkers (Ihle *et al.* 1981; Hapel *et al.* 1981) and designated Interleukin 3 (IL3). IL3 is defined by its ability to induce the enzyme 20 alpha-hydroxy-steroid dehydrogenase in nude mouse spleen cells. It also allows the establishment of cloned T cells which are Lyt 1^+, 2^- (helper) T cells. These helper T cells produce IL2 when stimulated with PMA (Hapel *et al.* 1981). The DEAE run-through material has not been assayed for its ability to induce 20 alpha-hydroxysteroid dehydrogenase, but it does stimulate the continuous proliferation of cells which are morphologically distinct from those grown in IL2. Preliminary experiments indicate that these cells do produce IL2 when stimulated with PMA (C. Havele, unpublished observations), as do the cells grown in IL3 (Hapel *et al.* 1981). IL3 is similar to the DEAE run-through material in terms of its molecular radius and its behaviour during ion-exchange chromatography. IL3, however, does not have any TCGF activity as shown by Ihle *et al.*(1981). Final proof that the DEAE run-through material is IL3 awaits further experiments.

When the IL2 produced by Con A-stimulated spleen cells was fractionated by DEAE-Sephacel chromatography, no activity was observed in the run-through fraction. This lack of putative IL3 (which is produced by spleen cells stimulated with Con A [Ihle *et al.* 1981]) may simply reflect a much lower level of production than is observed with EL4 lymphoma cells.

The relationship between IL2 and IL3 is unclear. Although functionally and physico-chemically different, these two lymphokines do have features in common, including (1) their production by Con A-stimulated spleen cells, (2) their molecular radius and (3) their ability to stimulate the continuous proliferation of T cells.

Two further fractionations were used to characterize fraction 6 IL2 - gel filtration chromatography after SDS denaturation, and chromatofocusing. The fractionation of SDS-denatured IL2 by gel filtration gave similiar results to those obtained with the IL2 from Con A-stimulated spleen cells. SDS-denatured IL2 behaved like a protein of 16,000 MW (Figure 17) suggesting that native IL2 may be composed of 2 subunits of equal or identical MW. Figure 17 also illustrates that the majority of protein present in fraction 6 material had a MW of approximately 67,000, even though this preparation had been chromatographed twice over Sephadex G-100.

Fraction 6 IL2 was characterized by chromatofocusing and a heterogeneity in pI values was observed (Figure 15). The IL2 had pI values ranging from 4.6 to less than 3.4. The pI values obtained with chromatofocusing are slightly lower than the actual pI values as would be determined with isoelectric focusing. Proteins elute from the chromatofocusing column at a pH slightly higher than their pI due to electrostatic interactions between the protein and the anion exchange resin. A heterogeneity in the isoelectric profile of IL2 from Con A-stimulated spleen cells has been shown by several investigators (Shaw *et al.* 1978b; Watson *et al.* 1979a,b; Farrar, J.J. *et al.* 1980a). In our laboratory, Dr. J. Shaw demonstrated that conventional isoelectric focusing resolved IL2 into several peaks of activity with a major peak at pI 3.8 and activity at pI values ranging from 4.0 to 5.0. At best, 3 separate species of IL2 were detected. IL2 produced by the EL4 lymphoma cells has also been characterized by conventional isoelectric focusing (Farrar, J.J. *et al.* 1980a). The IL2 was resolved into 3 isoelectric forms with pI values between 3.8 and 4.4. Chromato-focusing resolved IL2 into 10 different forms, a dramatic increase in resolution. In

addition, recovery of activity was typically in the range of 70 to 80% in contrast to the 40–50% obtained with isoelectric focusing. Some of the heterogeneity observed in IL2 by isoelectric focusing reflects a different pattern of glycosylation. Farrar has shown that treatment of IL2 with neuraminidase reduces this heterogeneity to a single form with a pI value of 4.9 (J. Farrar, personal communication).

The IL2 which eluted from the chromatofocusing column with the 1 M NaCl wash had a specific activity of 2000 units/microgram protein. This value can be used to determine the potency of IL2. The TCGF assay can detect 0.1 unit/ml IL2 (3% maximum stimulation). If all of the protein present in this 1 M NaCl sample were IL2, the TCGF assay was detecting 0.05 nanograms / ml IL2. Based on a MW of 16,000, 0.05 nanograms/ml represents approximately 3×10^{-12} M IL2. The 1 M NaCl wash material was not, however, homogeneous. SDS–PAGE showed several protein bands of approximately 20,000 MW (which may represent IL2) and 1 band at approximately 70,000 MW (data not shown). The 70,000 MW protein represented about 2/3 of the protein present in the sample as estimated from the intensity of staining after the PAGE. Pure IL2 therefore would have approximately 6,000 TCGF units/microgram of protein and be active at 10^{-12} M. Farrar has recently estimated that homogeneous IL2 is active at approximately 10^{-12} M (J. Farrar, personal communication).

An estimate of what percentage IL2 represents in terms of the total protein produced by EL4 cells can be made. We have observed that 1×10^9 EL4 lymphoma cells weigh approximately 1 gram. If 10% of this weight is protein, 10^8 cells produce about 0.1 mg of protein. The 2000 units of IL2 produced by 10^6 cells represents 0.3 micrograms of protein (based on purity being 6,000 units/microgram). Thus approximately 0.3% of the total protein is IL2. The observation that the EL4 lymphoma cells stop proliferating in response to PMA and therefore stop producing structural protein, suggests that this percentage might be significantly underestimated. Even so, at least 0.3% total protein and therefore probably 0.3% of the total mRNA is IL2. The isolation of a clone of mRNA for IL2, after insertion into a plasmid as complementary DNA, should therefore require screening through less than 300 bacterial colonies. mRNA purified by size would significantly increase this frequency. An attempt to isolate a clone of DNA for IL2 is underway in our laboratory.

V. CONCLUDING DISCUSSION

The results presented in this thesis establish several biochemical characteristics of IL2 and detail a convenient, reproducible protocol for its partial purification. Murine IL2: (1) has a molecular radius corresponding to globular proteins of approximately 30,000–45,000 MW; (2) is an acidic molecule which at pH 7.3 elutes from DEAE–Sephacel at a salt concentration of 0.12 to 0.14 M NaCl; (3) can be fractionated by hydrophobic interaction chromatography on Phenyl–Sepharose, thus separating IL2 from colony stimulating factor; and (4) is heterogeneous with regard to pI with values ranging from 4.6 to less than 3.4. These characteristics, among others, provide a basis for comparing IL2 with other immunostimulatory lymphokines. Through this comparison, one can begin to resolve which activities observed in the crude supernatants of stimulated lymphoid cells are due to IL2 and which are due to other lymphokines.

The supernatants of Con A–activated spleen cells are presently known to contain, in addition to IL2, (1) IL1 (Farrar, J.J. *et al.* 1978), (2) IL3 (Ihle *et al.* 1981), (3) interferon (Simon *et al.* 1979), (4) 'late–acting' TRF (Wecker *et al.*, 1975), (5) 'B cell growth factor' (J. Farrar, personal communication) and (6) colony stimulating factor (Hilfiker *et al.* 1981). The recognition that these factors represent unique entities is a result of being able to characterize them biochemically, and the development of well–defined assay systems in which these factors are active.

In addition to defining several biochemical characteristics of IL2, this purification protocol provides a convenient and reproducible procedure for isolating IL2 from other lymphokines present in crude supernatants. This protocol may provide the basis for purifying IL2 to homogeneity. The purest preparation of IL2 indicated that activity was detectable at less than 3×10^{-13} M with the only detectable impurity being horse serum albumin. Thus, a highly specific interaction occurs between IL2 and its receptor. It is only through the use of homogeneous preparations of IL2 that the role of this lymphokine in the immune system can be defined in molecular terms.

IL2 is produced from Con A–stimulated spleen cells and from a variant of EL4 lymphoma cells stimulated with the tumor promoter PMA (Chapter IV, Farrar, J.J. *et al.* 1980). The IL2 produced from the EL4 lymphoma cells is biochemically indistinguishable from that produced by murine spleen cells. The EL4 lymphoma cells produce

approximately 250 fold more IL2 on a per cell basis than do Con A-stimulated spleen cells. These cells, therefore, are an excellent source of IL2 for biological experiments as well as for biochemical characterization. In addition, these cells are a source of IL2 mRNA. This mRNA has been translated in vitro into biologically active IL2 (Bleackley *et al*. 1981). The subsequent cloning and isolation of the genetic material for IL2 should provide a probe for studying the regulation of IL2 production on a genetic level, as well as providing a source of IL2 absolutely free of any other eukaryotic proteins.

The biological activity used to monitor IL2 during the various chromatographic procedures was the proliferation of antigen-activated T cells. Two different assays, the costimulator assay and the TCGF assay, gave essentially identical results. The T cells used in the TCGF assay are a homogeneous source of T cells which can be used in studying immune activation and regulation at a clonal level. These cells may be valuable in defining the role of T cells in the immune response, as B cell myelomas have been in defining the role of B cells.

The recovery of IL2 activity after SDS-denaturation has allowed further characterization of this lymphokine. SDS-denatured IL2 has a MW of 16,000 as determined by gel filtration chromatography and glycerol gradient centrifugation. This provides a MW value independent of the shape of the molecule (Siegel and Monty 1966). Native murine IL2 exists as a protein of 31,000 MW (Shaw *et al*. 1978b), approximately twice that of SDS-denatured IL2. With the MW of 16,000 for SDS-denatured murine IL2, all 3 species of IL2 studied so far - rat, human and murine - can exist as polypeptide chains of 15,000 to 16,000 MW (Chapter III; Gillis *et al*. 1980b). Both rat and human IL2 will stimulate the continuous proliferation of murine T cells (Gillis *et al*. 1980b), but murine IL2 is inactive in stimulating human T cells. Thus, there appears to be some conservation during evolution of the molecule responsible for IL2 activity in terms of structure and function, as indicated by the cross-reactivity observed in the assays.

A monoclonal antibody with specificty against IL2 has been described (Gillis and Henney 1981; Gillis *et al*. 1981). This antibody inhibits the ability of IL2 to (1) stimulate the continuous proliferation of T cells and (2) to stimulate the differentiation of CTLs against alloantigen. It should be a powerful tool in defining the role of IL2 in immune regulation in vitro and in vivo. It may also prove to be valuable in screening the products of DNA clones

for IL2 determinants. Although generated against murine IL2, the monoclonal antibody cross-reacts with both human and rat IL2, thus it recognizes a determinant common to all 3 species (Gillis and Henney 1981; Gillis *et al*. 1981). A clone of DNA for IL2 obtained from mouse cells will therefore probably serve as a probe in both the human and rat systems.

REFERENCES

Aarden, L.A., *et al*. 1979. Revised nomenclature for antigen nonspecific T cell proliferation and helper factors. J. Immunol., **123**: 2928-2929.

Andersson, J., and F. Melchers. 1981. T cell-dependent activation of resting B cells: Requirement for both nonspecific unrestricted and antigen-specific Ia-restricted soluble factors. Proc. Nat. Acad. Sci. USA **78**: 2497-2501.

Andrews, P. 1970. Estimation of molecular size and molecular weights of biological compounds by gel filtration. Methods of Biochem. Anal. **18**: 1-53.

Askonas, B.A., A. Schimpl, and E. Wecker. 1974. The differentiation function of T cell-replacing factor in nu/nu spleen cell cultures. Eur. J. Immunol., **4**: 164-169.

Bach, F.H., B.J. Alter, S. Solliday, D.C. Zoschke, and M. Janis. 1970. Lymphocyte reactivity in vitro II. Soluble reconstituting factor permitting response of purified lymphocytes. Cell. Immunol., **1**: 219-227.

Bach, F.H., and K. Hirschlorn. 1964. Lymphocyte interaction: A potential histocompatibility test in vitro. Science, **143**: 813-814.

Bain, B., M.R. Vas, and L. Lowenstein. 1964. The development of large immature mononuclear cells in mixed leukocyte cultures. Blood, **23**: 108-116.

Beller, D.I., and E.R. Unanue. 1977. Thymic maturation in vitro by a secretory product from macrophages. J. Immunol., **118**: 1780-1787.

Benacerraf, B. 1981. Role of MHC gene products in immune regulation. Science, **212**: 1229-1238.

Bleackley, R.C., B. Caplan, C. Havele, R.G. Ritzel, T.R. Mosmann, J.J. Farrar, and V. Paetkau. 1981. Translation of lymphocyte mRNA into biologically-active interleukin 2 in oocytes. J. Immunol., **127**: 2432-2435.

Bleackley, R.C., C. Havele, and V. Paetkau. 1982. Cellular and molecular properties of an antigen-specific cytotoxic T lymphocyte line. J. Immunol., (in press).

Blyden, G., and R.E. Handschumacher. 1977. Purification and properties of human lymphocyte activating factor (LAF). J. Immunol., **118**: 1631-1638.

Bonnard, G.D., K. Yasaka, and D. Jacobson. 1979. Ligand-activated T cell growth factor-induced proliferation: Absorption of T cell growth factor by activated T cells. J. Immunol., **123**: 2704-2708.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities utilizing the principal of protein-dye binding. Anal. Biochem., 72: 248-254.

Bretscher, P., and M. Cohn. 1970. A theory of self-nonself discrimination. Science, 169: 1042-1049.

Burgess, A.W., D. Metcalf,and S. Russell. 1978. Regulation of hematopoietic differentiation and proliferation by colony stimulating factors. In Differentation of Normal and Neoplastic Hematopoietic Cells. (B. Clarkson, P.A. Marks, and J.E. Till, eds.) Cold Spring Harbor Labs., pp. 339-357.

Burnet, M. 1959. The clonal selection theory of acquired immunity. Vanderbilt University Press. Nashville, Tennessee.

Calderon, J., and E.R. Unanue. 1975. Two biological activities regulating cell proliferation found in cultures of peritoneal exudate cells. Nature, 253: 359-361.

Cantor, H., and E.A. Boyse. 1975. Functional subclasses of T lymphocytes bearing different Ly antigens. II. Cooperation between subclasses of Ly$^+$ cells in the generation of killer activity. J. Exp. Med., 141: 1390-1399.

Cantor, H., and E.A. Boyse. 1977. Lymphocytes as models for the study of mammalian cellular differentiation. Immunol. Rev., 33: 105-124.

Chen, D-M., and G. DiSabato. 1976. Further studies on the thymocyte stimulating factor. Cell. Immunol., 22: 211-224.

Chen, D-M., and G. DiSabato. 1977. Some effects of thymocyte-stimulating factor. Cell. Immunol., 30: 195-203.

Claman, H.N., E.A. Chaperon, and R.F. Triplett. 1966. Thymus-marrow cell combinations. Synergism in antibody production. Proc. Soc. Exp. Med., 122: 1167-1171.

Coutinho, A., E.L. Larsson, K.O. Gronvik, and J. Andersson. 1979. Studies on T lymphocyte activation. II. The target cells for Concanavalin A-induced growth factors. Eur. J. Immunol., 9: 587-592.

Delovitch, T.L., and M.L. Phillips. 1981. The biological and biochemical basis of allogeneic effect factor (AEF) activity: Relationship to T cell alloreactivity. Immunobiology, 159 (in press).

Delovitch, T.L., J. Watson, R. Battistella, J.F. Harris, J. Shaw, and V. Paetkau. 1981. In vitro analysis of allogeneic lymphocyte interaction. V. Identification and characterization

of two components of allogeneic effect factor, one of which displays H-2 restricted helper activity and the other, T cell-growth factor activity. J. Exp. Med., **153**: 107-128.

Dennert, G. 1980. Cloned lines of natural killer cells. Nature, **287**: 47-49.

Diamantstein, T., and A. Ulmer. 1975. Stimulation by cyclic GMP of lymphocytes mediated by soluble factor released from adherent cells. Nature, **256**: 418-419.

DiSabato, G., D.M.Chen, and M. Altin. 1978. Production of thymocyte stimulating factor by murine spleen lymphocytes: Cellular and biochemical mechanisms. Cell. Immunol., **35**: 340-352.

DiSabato, G., D.M. Chen, J.W. Erickson. 1975. Production by murine spleen cells of an activity stimulating the PHA responsiveness of thymus lymphocytes. Cell. Immunol., **17**: 495-504.

Doria, G., G. Agarossi, and S. DiPietro. 1972. Enhancing activity of thymocyte culture cell-free medium on the in vitro immune response of spleen cells from neonatally thymectomized mice to sheep RBC. J. Immunol., **108**: 268-270.

Dumonde, D.C., R.A. Wolstencroft, G.S. Panayi, M. Matthew, J. Morley, and W.T. Hawson. 1969. "Lymphokines": Non-antibody mediators of cellular immunity generated by lymphocyte activation. Nature, **224**: 38-42.

Dutton, R.W. 1975. Separate signals for the initiation of proliferation and differentiation in the B cell response to antigen. Transplant. Rev., **23**: 66-77.

Dutton, R.W., R. Falkoff, J.A. Hirst, M. Hoffmann, J.W. Kappler, J.R. Kettman, J.F. Lesley, and D. Vann. 1971. Is there evidence for a non-specific diffusable chemical mediator from the thymus-derived cell in the initiation of the immune response? Progress in Immunology, 1: 355-368. (B. Amos, ed.) Associated Press N.Y.

Economou, J.S., and H.S. Shin. 1978. Lymphocyte-activating factor. I. Generation and physiochemical characterization. J. Immunol., **121**: 1446-1452.

Eshhar, Z., R.N. Apte, I. Lowy, Y. Ben-Neriah, D. Givol, and E. Mozes. 1980. T-cell hybridoma bearing heavy chain variable region determinants producing (T,G)-A--L-specific helper factor. Nature, **286**: 270-272.

Farr, A.G., M.E. Dorf, and E.R. Unanue. 1977. Secretion of mediators following T lymphocyte-macrophage interaction is regulated by the major histocompatibility

complex. Proc. Nat. Acad. Sci. USA, **74**: 3542–3546.

Farrar, J.J., and J. Fuller–Bonar. 1976. The xenogeneic effect. II. Requirement for unactivated murine T cells during restoration of immune responsiveness with xenogeneic reconstitution factor. J. Immunol., **117**: 274–280.

Farrar, J.J., J. Fuller–Farrar, P.L. Simon, M.L. Hilfiker, B.M. Stadler, and W.L. Farrar. 1980a. Thymoma production of T cell growth factor (Interleukin 2). J. Immunol., **125**: 2555–2558.

Farrar, J.J., W.J. Koopman, and J. Fuller–Bonar. 1977. Identification and partial purification of two synergistically acting helper mediators in human mixed leukocyte culture supernatants. J. Immunol., **119**: 47–54.

Farrar, J.J., S.B. Mizel, J. Fuller–Farrar, W.L. Farrar, and M.L. Hilfiker. 1980b. Macrophage–independent activation of helper T cells. I. Production of Interleukin 2. J. Immunol., **125**: 793–798.

Farrar, J.J., P.L. Simon, W.J. Koopman, and J. Fuller–Bonar. 1978. Biochemical relationship of thymocyte mitogenic factor and factors enhancing humoral and cell–mediated immune responses. J. Immunol., **121**: 1353–1360.

Farrar, W.L., H.M. Johnson, and J.J. Farrar. 1981. Regulation of the production of immune interferon and cytotoxic T lymphocytes by Interleukin 2. J. Immunol., **126**: 1120–1125.

Farrar, W.L., S.B. Mizel, and J.J. Farrar. 1980. Participation of lymphocyte activating factor (Interleukin 1) in the induction of cytotoxic T cell responses. J. Immunol., **124**: 1371–1377.

Feldmann, M., and A. Basten. 1972. Cell interactions in the immune response in vitro IV. Comparison of the effects of antigen–sepcific and allogeneic thymus derived cell factors. J. Exp. Med., **136**: 722–736.

Finke, J.H., C.S. Orosz, and J.R. Battisto. 1977. Splenic T–killer cells can be generated by allogeneic thymic cells in conjunction with assisting factor. Nature, **267**: 353–354.

Germain, R.N., and B. Benacerraf. 1978. Antigen–specific T cell–mediated suppression. III. Induction of antigen specific suppressor T cells (Ts_3) in L–glutamic acid^{60}–L–alanine^{30}–L–tyrosine^{10} (GAT) responsive mice by nonresponder–derived GAT–suppressor factor (GAT–TsF). J. Immunol., **121**: 608–612.

Gery, I., R.K. Gershon, and B.H. Waksman. 1971. Potentiation of cultured mouse thymocyte responses by factors released by peripheral leukocytes. J. Immunol., **107**: 1778-1780.

Gery, I., R.K. Gershon, and B.H. Waskman. 1972. Potentiation of the T-lymphocyte response to mitogens. I. The responding cell. J. Exp. Med., **136**: 128-142.

Gery, I., and R.E. Handschumacher. 1974. Potentiation of the T lymphocyte response to mitogens. III. Properties of the mediator(s) from adherent cells. Cell. Immunol., **11**: 162-169.

Gery, I., and B.H. Waksman. 1972. Potentiation of the T-lymphocyte response to mitogens. II. The cellular source of potentiating mediator(s). J. Exp. Med., **136**: 143-155.

Gillis, S., P.E. Baker, F.W. Ruscetti, and K.A. Smith. 1978a. Long-term culture of human antigen specific cytotoxic T-cell lines. J. Exp. Med., **148**: 1093-1098.

Gillis, S., G.R. Crabtree, and K.A. Smith. 1979a. Glucocorticoid-induced inhibition of T cell growth factor production. I. The effect on mitogen-induced lymphocyte proliferation. J. Immunol., **123**: 1624-1631.

Gillis, S., G.R. Crabtree, and K.A. Smith. 1979b. Glucocorticoid-induced inhibition of T cell growth factor production. II. The effect on the in vitro generation of cytolytic T cells. J. Immunol., **123**: 1632-1638.

Gillis, S., M.M. Ferm, W. Ou, and K.A. Smith. 1978b. T cell growth factor: Parameters of production and a quantitative microassay for activity. J. Immunol., **120**: 2027-2032.

Gillis, S., A.E. Gillis, and C.S. Henney. 1981. Monoclonal antibody directed against interleukin 2. I. Inhibition of T lymphocyte mitogenesis and the in vitro differentiation of alloreactive cytolytic T cells. J. Exp. Med., **154**: 983-988.

Gillis, S., and C. Henney. 1981. The biochemical and biological characterization of lymphocyte regulatory molecules. VI. Generation of a B cell hybridoma whose antibody product inhibits interleukin 2 activity. J. Immunol., **126**: 1978-1984.

Gillis, S., and S.B. Mizel. 1981. T-cell lymphoma model for the analysis of interleukin 1-mediated T-cell activation. Proc. Nat. Acad. Sci. USA, **78**: 1133-1137.

Gillis, S., M. Scheid, and J. Watson. 1980a. Biochemical and biologic characterization of lymphocyte regulatory molecules. III. The isolation and phenotypic characterization of interleukin 2 producing T cell lymphomas. J. Immunol., **125**: 2570-2578.

Gillis, S., and K.A. Smith, 1977. Long term culture of tumor-specific cytotoxic T cells. Nature, **268**: 154-156.

Gillis, S., K.A. Smith, and J. Watson. 1980b. Biochemical characterization of lymphocyte regulatory molecules. II. Purification of a class of rat and human lymphokines. J. Immunol., **124**: 1954-1962.

Gillis, S., N.A. Union, P.E. Baker, and K.A. Smith. 1979c. The in vitro generation and substained culture of nude mouse cytolytic T lymphocytes. J. Exp. Med., **149**: 1460-1476.

Gillis, S., and J. Watson. 1981. Interleukin 2 induction of hapten-specific cytolytic T cells in nude mice. J. Immunol., **126**: 1245-1248.

Gisler, R.H., F. Staber, E. Rude, and P. Dukor. 1973. Soluble mediators of T-B interaction. Eur. J. Immunol., **3**: 650-652.

Gorczynski, R.M., R.G. Miller, and R.A. Phillips. 1972. Initiation of antibody production to sheep erythrocytes in vitro: Replacement of the requirement for T cells with a cell-free factor isolated from cultures of lymphoid cells. J. Immunol., **108**: 547-551.

Gorczynski, R.M., R.G. Miller, and R.A. Phillips. 1973a. Reconstitution of T cell depleted spleen cell populations by factors derived from T cells. I. Conditions for the production of active T cell supernatants. J. Immunol., **110**: 968-983.

Gorczynski, R.M., R.G. Miller, and R.A. Phillips. 1973b. Reconstitution of T cell-depleted spleen cell populations by factors derived from T cells. III. Mechanism of action of T cell-derived factors. J. Immunol., **111**: 900-913.

Gordon, J., and L.D. Maclean. 1965. A lymphocyte stimulating factor produced in vitro. Nature, **208**: 795-796.

Granelli-Piperno, A., J.D. Vassalli, and E. Reich. 1981. Purification of murine T cell growth factor. A lymphocyte mitogen with helper activity. J. Exp. Med., **154**: 422-431.

Greaves, M., and G. Janossy. 1972. Elicitation of selective T and B lymphocyte responses by cell surface binding ligands. Transp. Rev., **11**: 87-130.

Hapel, J.A., J.C. Lee, W.L. Farrar, and J.N. Ihle. 1981. Establishment of continuous cultures of Thy 1.2$^+$, Lyt 1$^+$, 2$^-$ T cells with purified interleukin 3. Cell, **25**: 179-186.

Hardt, C., M. Rollinghoff, K. Pfizenmaier, H. Mosmann, and H. Wagner. 1981. Lyt 23$^+$ cyclophosphamide-sensitive T cells regulate the activity of an interleukin 2 inhibitor

in vivo. J. Exp. Med., 154: 262-274.

Harwell, L., J.W. Kappler, and P. Marrack. 1976. Antigen specific and non-specific mediators of T cell/B cell cooperation. III. Characterization of the non-specific mediator(s) from different sources. J. Immunol., 116: 1379-1384.

Harwell, L., B. Skidmore, P. Marrack, and J. Kappler. 1980. Concanavalin A - inducible, interleukin 2 producing T cell hybridoma. J. Exp. Med., 152: 893-904.

Henney, C.S., K. Kuribayashi, D.E. Kern, and S. Gillis. 1981. Interleukin 2 augments natural killer cell activity. Nature, 291: 335-338.

Hilfiker, M.L., R.N. Moore, and J.J. Farrar. 1981. Biologic properties of chromatographically separated murine thymoma- derived interleukin 2 and colony-stimulating factor. J. Immunol., 127: 1983-1987.

Hiramatsu, K., S. Miyatani, M. Kim, S. Yamada, K. Okumura, and T. Tada. 1981. Unique T cell Ia antigen expressed on a hybrid cell line producing antigen specific augmenting T cell factor. J. Immunol., 127: 1118-1122.

Hoffmann, M.K. 1980. Macrophages and T cells control distinct phases of B cell differentiation in the humoral immune response in vitro. J. Immunol., 125: 2076-2081.

Hoffmann, M., and R.W. Dutton. 1971. Immune response restoration with macrophage culture supernatants. Science, 172: 1047-1048.

Hoffmann, M.K., S. Koenig, R.S. Mittler, H.F. Oettgen, P. Ralph, C. Galanos, and U. Hammerling. 1979. Macrophage factor controlling differentiation of B cells. J. Immunol., 122: 497-502.

Hoffmann, M.K., and J. Watson. 1979. Helper T cell replacing factors secreted by thymus derived cells and macrophages: Cellular requirements for B cell activation and synergistic properties. J. Immunol., 122: 1371-1375.

Howie, S., C.R. Parish, C.S. David, I.F.C. McKenzie, P.H. Maurer, and M. Feldmann. 1979. Serological analysis of antigen specific helper factors specific for poly L (Tyr, Glu)-poly-DL ALA--poly-L Lys [(T,G)-A--L] and L GLU^{60} -L ALA^{30}-L TYR^{10} (GAT). Eur. J. Immunol., 9: 501-506.

Hubner, L., G. Muller, A. Schimpl, and E. Wecker. 1978. Partial characterization and purification of murine T cell replacing factor, TRF. II. Biochemical characteristics.

Immunochemistry, **15**: 33-39.

Hunig, T., A. Schimpl, and E. Wecker. 1974. Autoradiographic studies on the proliferation of antibody producing cells in vitro. J. Exp. Med., **139**: 754-760.

Hunig, T.H., A. Schimpl, and E. Wecker. 1977a. Mechanism of T cell help in the immune response to soluble protein antigens. I. Evidence for in situ generation and action of T cell replacing factor during the anamnestic response to dinitrophenyl keyhole limpet hemocyanin in vitro. J. Exp. Med., **145**: 1216-1227.

Hunig, T.H., A. Schimpl, and E. Wecker. 1977b. Mechanism of T cell help in the immune response to soluble protein antigens. II. Reconstitution of primary and secondary in vitro immune responses to dinitrophenyl carrier conjugates by T cell replacing factor. J. Exp. Med., **145**: 1228-1236.

Hunter, P., and J.R. Kettman. 1974. Mode of action of a supernatant activity from T cell cultures that nonspecifically stimulates the humoral immune response. Proc. Nat. Acad. Sci. USA, **71**: 512-516.

Ihle, J.N., L. Pepersack, and L. Rebar. 1981. Regulation of T cell differentiation: In vitro induction of 20 alpha-hydroxysteroid dehydrogenase in splenic lymphocytes from athymic mice by an unique lymphokine. J. Immunol., **126**: 2184-2189.

Janis, M., and F. Bach. 1970. Potentiation of in vitro lymphocyte reactivity. Nature, **225**: 238-239.

Kasakura, S., and L. Lowenstein. 1965. A factor stimulating DNA synthesis derived from the medium of leukocyte cultures. Nature, **208**: 794-795.

Katz, D.H. 1977. Lymphocyte Differentiation, Recognition and Regulation. Academic Press, New York.

Keller, D.M., J.E. Swierkosz, P. Marrack, and J.W. Kappler. 1980. Two types of functionally distinct, synergizing helper T cells. J. Immunol., **124**: 1350-1359.

Kern, D.E., S. Gillis, M. Okada, and C.S. Henney. 1981. The role of interleukin 2 (IL2) in the differentiation of cytotoxic T cells: The effect of monoclonal anti-IL2 antibody and absorption with IL2 dependent T cell lines. J. Immunol., **127**: 1323-1328.

Klein, J., A. Juretic, C.N. Baxevanis, and Z.A. Nagy. 1981. The traditional and a new version of the mouse H-2 complex. Nature, **291**: 455-460.

Kohler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of

predefined specificity. Nature, **256**: 495–497.

Kontiainen, S., and M. Feldmann. 1978. Suppressor cell induction in vitro. IV. Target of antigen specific suppressor factor and its genetic relationships. J. Exp. Med., **147**: 110–122.

Kontiainen, S., E. Simpson, E. Bohrer, P.C.L. Beverley, L.A. Herzenberg, W.C. Fitzpatrick, P. Vogt, A. Torano, I.F.C. McKenzie, and M. Feldmann. 1978. T cell lines producing antigen specific suppressor factor. Nature, **274**: 477–480.

Koopman, W.J., J.J. Farrar, and J. Fuller-Bonar. 1978. Evidence for the identification of lymphocyte activating factor as the adherent cell derived mediator responsible for enhanced antibody synthesis by nude mouse spleen cells. Cell. Immunol., **35**: 92–98.

Koppman, W.J., J.J. Farrar, J.J. Oppenheim, J. Fuller-Bonar, and S. Dougherty. 1977. Association of a low molecular weight helper factor(s) with thymocyte proliferative activity. J. Immunol., **119**: 55–60.

Kuribayashi, K., S. Gillis, D.E. Kern, and C.S. Henney. 1981. Murine N.K. cell cultures: Effects of interleukin 2 and interferon on cell growth and cytotoxic reactivity. J. Immunol., **126**: 2321–2327.

Lachman, L.B., M.P. Hacker, G.Y. Blyden, and R.E. Handschumacher. 1977a. Preparation of lymphocyte activating factor from continuous murine macrophage cell lines. Cell. Immunol., **34**: 416–419.

Lachman, L.B., M.P. Hacker, and R.E. Handschumacher. 1977b. Partial purification of human lymphocyte activating factor (LAF) by ultrafiltration and electrophoretic techniques. J. Immunol., **119**: 2019–2023.

Lachman, L.B., J.O. Moore, and R.S. Metzgar. 1978. Preparation and characterization of lymphocyte activating factor from acute monocytic and myelomonocytic leukemia cells. Cell. Immunol., **41**: 199–206.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, **227**: 680–685.

Lafferty, K.J., H.S. Warren, J.A. Woolnough, and D.W. Talmage. 1978. Immunological induction of T lymphocytes: Role of antigen and the lymphocyte costimulator. Blood Cells, **4**: 395–404.

Lafferty, K.J., and J. Woolnough. 1977. The origin and mechanism of the allograft reaction.

Immunol. Rev., **35**: 231-262.

Larsson, E.L., N.N. Iscove, and A. Coutinho. 1980. Two distinct factors are required for induction of T cell growth. Nature, **283**: 664-666.

Lonai, P., J. Puri, S. Bitton, Y. Ben-Neriah, D. Givol, and G.J. Hammerling. 1981a. H-2 restricted helper factor secreted by cloned hybridoma cells. J. Exp. Med., **154**: 942-951.

Lonai, P., J. Puri, and G. Hammerling. 1981b. H-2 restricted antigen binding by a hybridoma clone that produces antigen specific helper factor. Proc. Nat. Acad. Sci. USA, **78**: 549-553.

Marrack (Hunter), P.C., and J.W. Kappler. 1975. Antigen specific and nonspecific mediators of T cell/B cell cooperation. I. Evidence for their production by different T cells. J. Immunol., **114**: 1116-1125.

McKenzie, I.E.C., and T. Potter. 1979. Murine lymphocyte surface antigens. Adv. Immunol., **27**: 179-338.

Mills, G., V. Monticone, and V. Paetkau. 1976. The role of macrophages in thymocyte mitogenesis. J. Immunol., **117**: 1325-1330.

Mills, G.B., and V. Paetkau. 1980. Generation of cytotoxic lymphocytes to syngeneic tumors using co-stimulator (interleukin 2). J. Immunol., **125**: 1897-1903.

Mishell, R.I., and R.W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. J. Exp. Med., **126**: 423-442.

Mitchell, G.F., and J.F.A.P. Miller. 1968. Cell to cell interactions in the immune response. II. The source of hemolysin forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. J. Exp. Med., **128**: 821-837.

Mitchison, N.A. 1971. The carrier effect in the secondary response to hapten protein conjugates. II. Cellular cooperation. Eur. J. Immunol., **1**: 18-27.

Mizel, S.B. 1979. Physiochemical characterization of lymphocyte activating factor (LAF). J. Immunol., **122**: 2167-2172.

Mizel, S.B., and A. Ben-Zui. 1980. Studies on the role of lymphocyte activating factor (interleukin 1) in antigen induced lymph node lymphocyte proliferation. Cell. Immunol., **54**: 382-389.

Mizel, S.B., and D. Mizel. 1981. Purification to apparent homogeneity of murine interleukin

1. J. Immunol., **126**: 834-837.

Mizel, S.B., J.J. Oppenheim, and D.L. Rosenstreich. 1978a. Characterization of lymphocyte activating factor (LAF) produced by the macrophage cell line, P388D_1. I. Enhancement of LAF production by activated T lymphocytes. J. Immunol., **120**: 1497-1503.

Mizel, S.B., J.J. Oppenheim, and D.L. Rosenstreich. 1978b. Characterization of lymphocyte activating factor (LAF) produced by a macrophage cell line, P338D_1. II. Biochemical characterization of LAF induced by activated T cells and LPS. J. Immunol., **120**: 1504-1508.

Mizel, S.B., and D.L. Rosenstreich. 1979. Regulation of lymphocyte activating factor (LAF) production and secretion in P388D_1 cells: Identification of high molecular weight precursors of LAF. J. Immunol., **122**: 2173-2179.

Mizel, S.B., D.L. Rosenstreich, and J.J. Oppenheim. 1978c. Phorbol myristic acetate stimulates LAF production by the macrophage cell line, P388D_1. Cell. Immunol., **40**: 230-235.

Mochizuki, D., J. Watson, and S. Gillis. 1980a. Biochemical and biologic characterization of lymphocyte regulatory molecules. IV. Purification of interleukin 2 from a murine T cell lymphoma. J. Immunol., **125**: 2579-2583.

Mochizuki, D.Y., J. Watson, and S. Gillis. 1980b. Biochemical separation of interleukin 2. J. Immunol. Methods, **39**: 185-201.

Morgan, D.A., F.W. Ruscetti, and R. Gallo. 1976. Selective in vitro growth of T lymphocytes from normal human bone marrows. Science, **193**: 1007-1008.

Muller, G., L. Hubner, A. Schimpl, and E. Wecker. 1978. Partial characterization and purification of murine T cell replacing factor, TRF. I. Purification procedures and gel electrophoretic analysis. Immunochemistry, **15**: 27-32.

Muraguchi, A., T. Kishimoto, Y. Miki, T. Kuritani, T. Kaieda, K. Yoshizaki, and Y. Yamamura. 1981. T cell replacing factor (TRF) induced IgG secretion in a human B blastoid cell line and demonstration of acceptors for TRF. J. Immunol., **127**: 412-416.

Nabel, G., J.S. Greenberger, M.A. Sakakeeny, and H. Cantor. 1981. Multiple biologic activities of a cloned inducer T cell population. Proc. Nat. Acad. Sci. USA, **78**: 1157-1161.

North, J.R., J.T. Kemshead, and B.A. Askonas. 1977. Nonspecific factor replaces T cells in

an IgG response to soluble antigens. Immunol., **33**: 321-329.

Okada, M., T. Kishimoto, T. Igarashi, T. Teranishi, and Y. Yamamura. 1978. LPS- or 8Br-cyclic AMP induced production of T cell activating factor(s) in macrophage tumor cell line J774.1. J. Immunol., **120**: 1097-1101.

Okada, M., G.R. Klimpel, R.C. Kuppers, and C.S. Henney. 1979. The differentiation of cytotoxic T cells in vitro. I. Amplifying factor(s) in the primary response is Lyt 1^+ cell dependent. J. Immunol., **122**: 2527-2533.

Paetkau, V. 1981. Thymocyte stimulating factors. In The Lymphokines. Authors: Hadden/Steward. The Humana Press Inc., Clifton, New Jersey, pp. 181-205.

Paetkau, V., G. Mills, S. Gerhart, and V. Monticone. 1976. Proliferation of murine thymic lymphocytes in vitro is mediated by the Concanavalin A induced release of a lymphokine (costimulator). J. Immunol., **117**: 1320-1324.

Paetkau, V., J. Shaw, B. Caplan, G.B. Mills, and K.C. Lee. 1980a. Interleukin 2 in cell-mediated immune responses. J. Supramolecular Structure, **13**: 271-280.

Paetkau, V., J. Shaw, G. Mills, and B. Caplan. 1980b. Cellular origins and targets of costimulator (IL2). Immunol. Rev., **51**: 157-175.

Parker, D.C., and S. Prakash. 1981. The effect of culture supernate from an interleukin 2-producing cloned T cell hybridoma on anti-Ig activated B cells. In B Lymphocytes in the Immune Response: Functional, Developmental and Interactive Properties, pp. 193-199. Editors Klinman, Mosier, Scher and Vitetta. Elsevier North Holland.

Pickel, K., U. Hammerling, and M. Hoffmann. 1976. Ly phenotype of T cells releasing T cell replacing factor. Nature, **264**: 72.

Pierce, C.W., T. Tadakuma, and J.A. Kapp. 1979. Role of nonspecific and specific suppressor factors in immunity. Ann. N.Y. Acad. Sci., **332**: 336-344.

Pilarski, L.M. 1977. A requirement for antigen specific helper T cells in the generation of cytotoxic T cells from thymocyte precursors. J. Exp. Med., **145**: 709-725.

Pilarski, L.M. 1979. Antigen specific helper T cells are essential for cytotoxic T cell responses to metabolically inactivated stimulator cells. Eur. J. Immunol., **9**: 454-460.

Pilarski L.M., A. Al-Adra, and I.F.C. McKenzie. 1980. Surface markers on the T cells that regulate cytotoxic T cell responses. II. Distribution of Ly 6.1 and Ly 7.2 antigens on the effector cells of help and suppression. J. Immunol., **125**: 365-369.

Plate, J.M.D. 1976. Soluble factors substitute for T-T-cell collaboration in generation of T-killer lymphocytes. Nature, **260**: 329-331.

Plate, J.M.D. 1977. Two signals required for the initiation of killer T-cell differentiation. Cell. Immunol., **32**: 183-192.

Puri, J., M. Shinitzky, and P. Lonai. 1980. Concommitant increase in antigen binding and in T cell membrane lipid viscosity induced by the lymphocyte activating factor, LAF. J. Immunol., **124**: 1937-1942.

Reynolds, J.A., and C. Tanford. 1970a. Binding of dodecyl sulfate to proteins at high binding rates. Possible implications for the state of proteins in biological membranes. Proc. Nat. Acad. Sci. USA, **66**: 1002-1007.

Reynolds, J.A., and C. Tanford. 1970b. The gross conformation of protein sodium dodecyl sulfate complexes. J. Biol. Chem., **245**: 5161-5165.

Rosenwasser, L.J., and C.A. Dinarello. 1981. Ability of human leukocytic pyrogen to enhance phytohemagglutinin induced murine thymocyte proliferation. Cell. Immunol., **63**: 134-142.

Rulon, K., and D.W. Talmage. 1979. Enhancement of the primary cytotoxic response to membrane by a lymphokine costimulator. Proc. Nat. Acad. Sci. USA, **76**: 1994-1997.

Schimpl, A., and E. Wecker. 1972. Replacement of T cell function by a T cell product. Nature New Biol., **237**: 15-17.

Schrader, J.W., B. Arnold, and I. Clark-Lewis. 1980. A Con A-stimulated T cell hybridoma releases factors affecting haemotopoietic colony-forming cells and B-cell antibody responses. Nature, **283**: 197-199.

Schrader, J.W., and I. Clark-Lewis. 1981. T cell hybridoma-derived regulatory factors. I. Production of T cell growth factor following stimulation by Concanavalin A. J. Immunol., **126**: 1101-1105.

Segrest, J.P., and R.L. Jackson. 1972. Molecular weight determination of glycoproteins by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Methods in Enzymology, **28**: 54-63.

Segrest, J.P., R.L. Jackson, E.P. Andrews, and V.T. Marchesi. 1971. Human erythrocyte membrane glycoprotein: A re-evaluation of the molecular weight as determined by SDS polyacrylamide gel electrophoresis. Biochem. Biophys. Res. Commun., **44**:

390-395.

Shapiro, Al, E. Vinuela, and J.V. Maizel. 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem. Biophys. Res. Commun., **28**: 815-820.

Shaw, J., B. Caplan, V. Paetkau, L.M. Pilarski, T.L. Delovitch, and I.F.C. McKenzie. 1980. Cellular origins of costimulator (IL2) and its activity in cytotoxic T lymphocyte responses. J. Immunol., **124**: 2231-2239.

Shaw, J., V. Monticone, G. Mills, and V. Paetkau. 1978a. Effects of costimulator on immune responses in vitro. J. Immunol., **120**: 1974-1980.

Shaw, J., V. Monticone, and V. Paetkau. 1978b. Partial purification and molecular characterization of a lymphokine (costimulator) required for the mitogenic response of mouse thymocytes in vitro. J. Immunol., **120**: 1967-1973.

Shiozawa, C., M.B. Longenecker, and E. Diener. 1980. In vitro cooperation of antigen specific T cell derived helper factor B cells, and adherent cells or their secretory product in a primary IgM response to chicken MHC antigens. J. Immunol., **125**: 68-73.

Shiozawa, C., B. Singh, S. Rubenstein, and E. Diener. 1977. Molecular control of B cell triggering by antigen specific T cell-derived helper factor. J. Immunol., **118**: 2199-2205.

Siegel, L.M., and K.J. Monty. 1966. Determination of molecular weights and frictional ratios of proteins in impure systems by use of gel filtration and density gradient centrifugation. Application to crude preparations of sulfite and hydroxylamine reductases. Biochem. Biophys., Acta, **112**: 346-362.

Simon, P.L., J.J. Farrar, and P.D. Kind. 1979. Biochemical relationship between murine immune interferon and a killer cell helper factor. J. Immunol., **122**: 127-132.

Sjoberg, O., J. Andersson, and G. Moller. 1972. Reconstitution of the antibody response in vitro of T cell deprived spleen cells by supernatants from spleen cell cultures. J. Immunol., **109**: 1379-1385.

Smith, K.A. 1980. T cell growth factor. Immunol. Rev., **51**: 337-355.

Smith, K.A., K.J. Gilbride, and M.F. Favata. 1980a. Lymphocyte activating factor promotes T cell growth factor production by cloned murine lymphoma cells. Nature, **287**: 853-855.

Smith, K.A., S. Gillis, and P.E. Baker. 1979a. The role of soluble factors in the regulation of T cell immune reactivity. In The Molecular Bases of Immune Cell Function. J.G. Kaplan, editor, Elsevier-North Holland, Inc., New York, p. 223.

Smith, K.A., S. Gillis, P.E. Baker, and D. McKenzie. 1979b. T cell growth factor mediated T cell proliferation. Ann. N.Y. Acad. Sci., **332**: 423-432.

Smith, K.A., L.B. Lachman, J.J. Oppenheim, and M.F. Favata. 1980b. The functional relationship of the interleukins. J. Exp. Med., **151**: 1551-1556.

Stadler, B.M., S. Dougherty, J.J. Farrar, and J.J. Oppenheim. 1981. Relationship of cell cycle to recovery of IL2 activity from human mononuclear cells, human and mouse T cell lines. J. Immunol., **127**: 1936-1940.

Swain, S.L., G. Dennert, J.F. Warner, and R.W. Dutton. 1981. Culture supernatants of a stimulated T cell line have helper activity that acts synergistically with interleukin 2 in the response of B cells to antigen. Proc. Nat. Acad. Sci. USA, **78**: 2517-2521.

Tada, T., Hayakawa, K., K. Okumura, and M. Taniguchi. 1980. Coexistence of variable regions of immunoglobulin heavy chain and I region gene products on antigen specific suppressor T cells and suppressor T cell factor. A minimal model of functional antigen receptor of T cells. Mol. Immunol., **17**: 867-875.

Tada, T., M. Taniguchi, K. Hayakawa, and K. Okumura. 1979. Multiple MHC loci controlling lymphocyte interactions. In: T and B Lymphocytes: Recognition and Function. F.H. Bach, B. Bonavida, E.S. Vitetta, and C.F. Fox, editors. Academic Press. 1979. p. 293.

Takatsu, K., K. Tanaka, A. Tominaga, Y., Kumahara, and T. Hamaoka. 1980. Antigen-induced T cell replacing factor (TRF). III. Establishment of T cell hybrid clone continuously producing TRF and functional analysis of released TRF. J. Immunol., **125**: 2646-2653.

Talmage, D.W., J.A. Woolnough, H. Hemmingsen, L. Lopez, and K.J. Lafferty. 1977. Activation of cytotoxic T cells by nonstimulating tumor cells and spleen cell factor(s). Proc. Nat. Acad. Sci. USA, **74**: 4610-4614.

Taniguchi, M., T. Saito, and T. Tada. 1979. Antigen specific suppressive factor produced by a transplantable I-J bearing T cell hybridoma. Nature, **278**: 555-558.

Taniguchi, M., I. Takei, and T. Tada. 1980. Functional and molecular organization of an antigen specific suppressor factor from a T cell hybridoma. Nature, **283**: 227-228.

Taniguchi, M., and T. Tokumisa. 1980. Cellular consequences in the suppression of antibody

response by the antigen specific T-cell factor. J. Exp. Med., **151**: 517-527.

Taussig, M.J. 1974. T cell factor which can replace T cells in vivo Nature, **238**: 234-235.

Taussig, M.J. 1980. Antigen specific T cell factors. Immunol., **41**: 759-787.

Taussig, M.J., J.R.F. Corvalan, R.M. Binns, and A. Holliman. 1979a. Production of an H-2 related suppressor factor by a hybrid T cell line. Nature, **277**: 305-308.

Taussig, M.J., J.R.F. Corvalan, R.M. Binns, B. Roser, and A. Holliman. 1979b. Immunological activity of a T hybrid line. I. Production of an H-2 related suppressor factor with specificity for sheep red blood cells. Eur. J. Immunol., **9**: 768-776.

Taussig, M.J., J.R.F. Corvalan, and A. Holliman. 1979c. Characterization of an antigen specific factor from a hybrid T cell line. Ann. N.Y. Acad. Sci., **232**: 316-335.

Taussig, M.J., A. Holliman, and J.R.F. Corvalan. 1980. T cell hybrids as a source of antigen specific factors. Transplantation Proceedings, **XII**: 427-431.

Tokuhisa, T., M. Taniguchi, K. Okumura, and T. Tada. 1978. An antigen specific I region gene product that augments the antibody response. J. Immunol., **120**: 414-421.

Wagner, H. 1973. Cell mediated immune response in vitro IV: Metabolic studies on cellular immunogenicity. Eur. J. Immunol., **3**: 84-89.

Wagner, H., C. Hardt, K. Heeg, K. Pfizenmaier, W. Solbach, R. Bartlett, H. Stockingher, and M. Rollinghoff. 1980a. T-T cell interactions during cytotoxic T lymphocyte (CTL) responses: T cell derived helper factor (interleukin 2) as a probe to analyze CTL responsiveness and thymic maturation of CTL progenitors. Immunol. Rev., **51**: 215-255.

Wagner, H., C. Hardt, K. Heeg, M. Rollinghoff, and K. Pfizenmaier. 1980b. T-cell derived helper factor allows in vivo induction of cytotoxic T cells in nu/nu mice. Nature, **284**: 278-280.

Wagner, H., and M. Rollinghoff. 1978. T-T-cell interactions during in vitro cytotoxic allograft responses. I. Soluble products from activated Ly 1^+ T cells trigger autonomously antigen-primed Ly 23^+ T cells to cell proliferation and cytolytic activity. J. Exp. Med., **148**: 1523-1538.

Wagner, H., M. Rollinghoff, K. Pfizenmaier, C. Hardt, and G. Johnscher. 1980c. T-T-cell interactions during in vitro cytotoxic T lymphocyte (CTL) responses. II. Helper factor from activated Lyt 1^+ T cells is rate limiting; i) in T cell responses to nonimmunogenic

alloantigen, ii) in thymocyte responses to allogeneic stimulator cells, and iii) recruits allo-or H-2 restricted CTL precursors from the Lyt 123$^+$ T subset. J. Immunol., **124**: 1058-1067.

Wagner, H., M. Rollinghoff, R. Schawaller, C. Hardt, and K. Pfizenmaier, 1979. T cell derived helper factor allows Lyt 123 thymocytes to differentiate into cytotoxic T lymphocytes. Nature, **280**: 405-406.

Waksman, B.H., and Y. Namba. 1976. On soluble mediators of immunologic regulation. Cell. Immunol., **21**: 161-176.

Waldmann, H. 1975. T cell-dependent mediator in the immune response. III. The role of nonspecific factor (NSF) in the in vitro immune response. Immunol., **28**: 497-507.

Waldmann, H., and A. Munro. 1973. T cell-dependent mediator in the immune response. Nature, **243**: 356-357.

Waldmann, H., and A. Munro. 1974. T cell-dependent mediator in the immune response. II. Physical and biological properties. Immunol., **27**: 53-64.

Waldmann, H., P. Poulton, and C. Desaymard. 1976. Antigen nonspecific T cell factor in B cell activation. Origin, biological properties and failure to show a relationship to H-2. Immunol., **30**: 723-733.

Watson, J. 1973. The role of humoral factors in the initiation of in vitro primary immune responses. III. Characterization of factors that replace thymus derived cells. J. Immunol., **111**: 1301-1313.

Watson, J., L.A. Aarden, J. Shaw, and V. Paetkau. 1979a. Molecular and quantitative analysis of helper T cell replacing factors on the induction of antigen-sensitive B and T lymphocytes. J. Immunol., **122**: 1633-1638.

Watson, J., S. Gillis, J. Marbrook, D. Mochizuki, and K.A. Smith. 1979b. Biochemical and biological characterization of lymphocyte regulatory molecules. I. Purification of a class of murine lymphokines. J. Exp. Med., **150**: 849-861.

Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate polyacrylamide gel electrophoresis. J. Biol. Chem., **244**: 4406-4412.

Wecker, E., A. Schimpl, T. Hunig, and L. Huhn. 1975. A T-cell produced mediator substance active in the humoral immune response. Ann. N.Y. Acad. Sci., **249**: 258-263.

Weinberger, O., S. Herrmann, M.F. Mescher, B. Benacerraf, and S.J. Burakoff. 1981. Cellular

interactions in the generation of cytolytic T lymphocyte responses. Analysis of the helper T cell pathway. Eur. J. Immunol., 11: 405-411.

Wood, D.D. 1979a. Purification and properties of human B cell-activating factor. J. Immunol., 123: 2395-2399.

Wood, D.D. 1979b. Mechanism of action of human B cell activating factor. I. Comparison of the plaque stimulating activity with thymocyte stimulating activity. J. Immunol., 123: 2400-2407.

Zinkernagel, R.M., and P.C. Doherty. 1974. Restriction of in vitro T cell mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature, 248: 701-702.

PUBLICATIONS BASED WHOLLY OR PARTLY ON THIS WORK

Shaw, J., B. Caplan, V. Paetkau, L.M. Pilarski, T.L. Delovitch and I.F.C.McKenzie. 1980. Cellular origins of costimulator (IL2) and its activity in cytotoxic T lymphocyte responses. J. Immunol., **124**: 2231-2239.

Paetkau, V., J. Shaw, G. Mills, and B. Caplan. 1980. Cellular origins and targets of costimulator (IL2). Immunol. Revs., **51**: 157-175.

Paetkau, V., J. Shaw, B. Caplan, G.B. Mills, and K.-C. Lee. 1980. Interleukin-2 in cell-mediated immune responses. J. Supramol. Structure, **126**: 1351-1354.

Caplan, B., C. Gibbs, and V. Paetkau. 1981. Properties of sodium dodecyl sulfate-denatured interleukin-2. J. Immunol., **126**: 1351-1354.

Bleackley, R.C., B. Caplan, C. Havele, R.G. Ritzel, T.R. Mosmann, J.J. Farrar, and V. Paetkau. 1981. Translation of lymphocyte mRNA into biologically-active interleukin 2 in oocytes. J. Immunol., **127**: 2432-2435.